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Attorney Docket P1101P1  
PATENT

CERTIFICATION UNDER 37 CFR 1.10	
EM168889049 US: Express Mail Number	February 9, 1998: Date of Deposit
I hereby certify that this Non-provisional Application Transmittal and the documents referred to as enclosed therein are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner of Patents, Washington, D.C. 20231.	
 Janet Tse	

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BOX PATENT APPLICATION  
Assistant Commissioner of Patents  
Washington, D.C. 20231

**NON-PROVISIONAL APPLICATION TRANSMITTAL UNDER 37 CFR 1.53(b)**

Transmitted herewith for filing is a non-provisional patent application:

Inventor(s) (or Application "Identifier"):

Avi J. Ashkenazi  
Anan Chuntharapai  
Kyung Jin Kim

Title: Apo-2 Receptor

**1. Type of Application**

- ☐ This application is for an original, non-provisional application.
- ☐ This is a non-provisional application claiming priority to provisional application no. \_\_, filed \_\_, the entire disclosure of which is hereby incorporated by reference.
- ☒ This is a ☒ continuation-in-part ☐ continuation ☐ divisional application claiming priority to application Serial Number 08/857,216, filed May 15, 1997, the entire disclosure of which is hereby incorporated by reference.

**2. Papers Enclosed Which Are Required For Filing Date Under 37 CFR 1.53(b)  
(Non-provisional)**

80 pages of specification  
2 pages of claims  
1 page(s) of abstract  
12 sheet(s) of drawings  
☒ formal ☐ informal

### 3. Declaration or Oath

X An executed declaration of the inventor(s) ☐ is enclosed ☒ will follow.

\_\_\_\_ A copy of the executed declaration/oath filed in the prior application is enclosed (37 CFR 1.63(d)).

— A signed statement is attached deleting inventor(s) named in the prior application (see 37 CFR 1.63(d)(2) and 1.33(b)).

#### 4. Assignment

  X   An Assignment of the invention to GENENTECH, INC. [] is enclosed with attached Recordation Form Cover Sheet [X] will follow.

The prior application is assigned of record to Genentech, Inc.

## 5. Amendments (for continuation and divisional applications)

\_\_\_\_\_ Cancel in this application original claims \_\_\_ of the prior application before calculating the filing fee. (At least one original independent claim must be retained for filing purposes.)

\_\_\_\_\_ A preliminary amendment is enclosed. (Claims added by this amendment have been properly numbered consecutively beginning with the number next following the highest numbered original claim in the prior application.)

**Relate Back -- 35 U.S.C. 120 or 35 U.S.C. 119**

--This is a

continuation

X continuation-in-part

of co-pending application(s)

X Serial No. 08/857,216 filed on May 15, 1997, which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35

USC §120. --

\_\_\_\_ International Application \_ filed on \_\_\_\_ which designated the U.S., which application(s) is(are) incorporated herein by reference and to which application(s) priority is claimed under 35 USC §120.--

\_\_\_\_ provisional application No. \_ filed \_\_\_\_, the entire disclosure of which is hereby incorporated by reference and to which application(s) priority is claimed under 35 USC §119.--.

**6. Fee Calculation (37 CFR 1.16)**

The fee has been calculated as follows:

CLAIMS FOR FEE CALCULATION					
Number Filed		Number Extra		Rate	Basic Fee 37 CFR 1.16(a)
					\$790.00
Total Claims	18	- 20 =	0	X \$22.00	\$0.00
Independent Claims	10	- 3 =	7	X \$82.00	\$574.00
____ Multiple dependent claim(s), if any				+ \$270.00	\$0.00
Filing Fee Calculation					\$1,364.00

**7. Method of Payment of Fees**

The Commissioner is hereby authorized to charge Deposit Account No. 07-0630 in the amount of \$1,364.00. **A duplicate copy of this transmittal is enclosed.**

**8. Authorization to Charge Additional Fees**

The Commissioner is hereby authorized to charge any additional fees required under 37 CFR §1.16 and 1.17, or credit overpayment to Deposit Account No. 07-0630. **A duplicate copy of this sheet is enclosed.**

**9. Additional Papers Enclosed**

- ☐ Information Disclosure Statement (37 CFR §1.98) w/ PTO-1449 and citations
- ☐ Submission of "Sequence Listing", computer readable copy, certificate re: sequence listing, and/or amendment pertaining thereto for biological invention containing nucleotide and/or amino acid sequence.
- ☐ A new Power of Attorney or authorization of agent.
- ☐ Other:

**10. Maintenance of Copendency of Prior Application (for continuation and divisional applications)**

*[This item **must** be completed and the necessary papers filed in the prior application if the period set in the prior application has run]*

066020" 04.02.06.0

\_\_\_\_\_ A petition, fee and/or response has been filed to extend the term in the pending  
prior application until  
\_\_\_\_\_ A copy of the petition for extension of time in the *prior* application is attached.

**11. Correspondence Address:**

X Address all future communications to:

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Respectfully submitted,  
GENENTECH, INC.

Date: February 9, 1998

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PATENT DOCKET NO. P1101P1  
EXPRESS MAIL NO:EM168889049US  
MAILED: February 9, 1998

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Apo-2 Receptor

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RELATED APPLICATIONS

This application is a continuation-in-part application of United States Patent Application Serial No. 08/857,216 filed May 15, 1997, the contents of which are hereby incorporated by reference.

20

FIELD OF THE INVENTION

The present invention relates generally to the identification, isolation, and recombinant production of novel polypeptides, designated herein as "Apo-2".

25

BACKGROUND OF THE INVENTION

Apoptosis or "Programmed Cell Death"

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Control of cell numbers in mammals is believed to be determined, in part, by a balance between cell proliferation and cell death. One form of cell death, sometimes referred to as necrotic cell death, is typically characterized as a pathologic form of cell death resulting from some trauma or cellular injury. In contrast, there is another, "physiologic" form of cell death which usually proceeds in an orderly or controlled manner. This orderly or controlled form of cell death is often referred to as "apoptosis" [see, e.g., Barr et al., Bio/Technology, 12:487-493 (1994); Steller et al., Science, 267:1445-1449 (1995)]. Apoptotic cell death naturally occurs in many physiological processes, including embryonic development and clonal selection in the immune system [Itoh et al., Cell, 66:233-243 (1991)]. Decreased levels of apoptotic cell death have been associated with a variety of pathological conditions, including cancer, lupus, and herpes virus infection [Thompson, Science, 267:1456-1462 (1995)]. Increased levels of apoptotic cell death may be associated with a variety of other pathological conditions, including AIDS, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, retinitis pigmentosa, cerebellar degeneration, aplastic anemia, myocardial infarction, stroke, reperfusion injury, and toxin-induced

45

5 liver disease [see, Thompson, supra].

Apoptotic cell death is typically accompanied by one or more characteristic morphological and biochemical changes in cells, such as condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of  
10 mitochondrial function. A variety of extrinsic and intrinsic signals are believed to trigger or induce such morphological and biochemical cellular changes [Raff, Nature, 356:397-400 (1992); Steller, supra; Sachs et al., Blood, 82:15 (1993)]. For instance, they can be triggered by hormonal stimuli, such as glucocorticoid hormones for immature  
15 thymocytes, as well as withdrawal of certain growth factors [Watanabe-Fukunaga et al., Nature, 356:314-317 (1992)]. Also, some identified oncogenes such as *myc*, *rel*, and *ElA*, and tumor suppressors, like *p53*, have been reported to have a role in inducing apoptosis. Certain chemotherapy drugs and some forms of radiation have likewise been  
20 observed to have apoptosis-inducing activity [Thompson, supra].

#### TNF Family of Cytokines

Various molecules, such as tumor necrosis factor- $\alpha$  ("TNF- $\alpha$ "), tumor necrosis factor- $\beta$  ("TNF- $\beta$ " or "lymphotoxin"), CD30 ligand, CD27 ligand, CD40 ligand, OX-40 ligand, 4-1BB ligand, Apo-1 ligand (also  
25 referred to as Fas ligand or CD95 ligand), and Apo-2 ligand (also referred to as TRAIL) have been identified as members of the tumor necrosis factor ("TNF") family of cytokines [See, e.g., Gruss and Dower, Blood, 85:3378-3404 (1995); Wiley et al., Immunity, 3:673-682 (1995); Pitti et al., J. Biol. Chem., 271:12687-12690 (1996); WO 97/01633  
30 published January 16, 1997]. Among these molecules, TNF- $\alpha$ , TNF- $\beta$ , CD30 ligand, 4-1BB ligand, Apo-1 ligand, and Apo-2 ligand (TRAIL) have been reported to be involved in apoptotic cell death. Both TNF- $\alpha$  and TNF- $\beta$  have been reported to induce apoptotic death in susceptible tumor cells [Schmid et al., Proc. Natl. Acad. Sci., 83:1881 (1986); Dealtry et al.,  
35 Eur. J. Immunol., 17:689 (1987)]. Zheng et al. have reported that TNF- $\alpha$  is involved in post-stimulation apoptosis of CD8-positive T cells [Zheng et al., Nature, 377:348-351 (1995)]. Other investigators have reported that CD30 ligand may be involved in deletion of self-reactive T cells in the thymus [Amakawa et al., Cold Spring Harbor Laboratory Symposium on  
40 Programmed Cell Death, Abstr. No. 10, (1995)].

5 Mutations in the mouse Fas/Apo-1 receptor or ligand genes  
(called *lpr* and *gld*, respectively) have been associated with some  
autoimmune disorders, indicating that Apo-1 ligand may play a role in  
regulating the clonal deletion of self-reactive lymphocytes in the  
periphery [Krammer et al., Curr. Op. Immunol., 6:279-289 (1994); Nagata  
10 et al., Science, 267:1449-1456 (1995)]. Apo-1 ligand is also reported  
to induce post-stimulation apoptosis in CD4-positive T lymphocytes and  
in B lymphocytes, and may be involved in the elimination of activated  
lymphocytes when their function is no longer needed [Krammer et al.,  
supra; Nagata et al., supra]. Agonist mouse monoclonal antibodies  
15 specifically binding to the Apo-1 receptor have been reported to exhibit  
cell killing activity that is comparable to or similar to that of TNF- $\alpha$   
[Yonehara et al., J. Exp. Med., 169:1747-1756 (1989)].

#### TNF Family of Receptors

Induction of various cellular responses mediated by such TNF  
20 family cytokines is believed to be initiated by their binding to  
specific cell receptors. Two distinct TNF receptors of approximately  
55-kDa (TNFR1) and 75-kDa (TNFR2) have been identified [Hohman et al.,  
J. Biol. Chem., 264:14927-14934 (1989); Brockhaus et al., Proc. Natl.  
Acad. Sci., 87:3127-3131 (1990); EP 417,563, published March 20, 1991]  
25 and human and mouse cDNAs corresponding to both receptor types have been  
isolated and characterized [Loetscher et al., Cell, 61:351 (1990);  
Schall et al., Cell, 61:361 (1990); Smith et al., Science, 248:1019-1023  
(1990); Lewis et al., Proc. Natl. Acad. Sci., 88:2830-2834 (1991);  
Goodwin et al., Mol. Cell. Biol., 11:3020-3026 (1991)]. Extensive  
30 polymorphisms have been associated with both TNF receptor genes [see,  
e.g., Takao et al., Immunogenetics, 37:199-203 (1993)]. Both TNFRs  
share the typical structure of cell surface receptors including  
extracellular, transmembrane and intracellular regions. The  
extracellular portions of both receptors are found naturally also as  
35 soluble TNF-binding proteins [Nophar, Y. et al., EMBO J., 9:3269 (1990);  
and Kohno, T. et al., Proc. Natl. Acad. Sci. U.S.A., 87:8331 (1990)].  
The cloning of recombinant soluble TNF receptors was reported by Hale et  
al. [J. Cell. Biochem. Supplement 15F, 1991, p. 113 (P424)].

The extracellular portion of type 1 and type 2 TNFRs (TNFR1  
40 and TNFR2) contains a repetitive amino acid sequence pattern of four  
cysteine-rich domains (CRDs) designated 1 through 4, starting from the

5 NH<sub>2</sub>-terminus. Each CRD is about 40 amino acids long and contains 4 to 6  
cysteine residues at positions which are well conserved [Schall et al.,  
supra; Loetscher et al., supra; Smith et al., supra; Nophar et al.,  
supra; Kohno et al., supra]. In TNFR1, the approximate boundaries of  
10 the four CRDs are as follows: CRD1- amino acids 14 to about 53; CRD2-  
amino acids from about 54 to about 97; CRD3- amino acids from about 98  
to about 138; CRD4- amino acids from about 139 to about 167. In TNFR2,  
CRD1 includes amino acids 17 to about 54; CRD2- amino acids from about  
55 to about 97; CRD3- amino acids from about 98 to about 140; and CRD4-  
15 amino acids from about 141 to about 179 [Banner et al., Cell, 73:431-435  
(1993)]. The potential role of the CRDs in ligand binding is also  
described by Banner et al., supra.

A similar repetitive pattern of CRDs exists in several other  
cell-surface proteins, including the p75 nerve growth factor receptor  
(NGFR) [Johnson et al., Cell, 47:545 (1986); Radeke et al., Nature,  
20 325:593 (1987)], the B cell antigen CD40 [Stamenkovic et al., EMBO J.,  
8:1403 (1989)], the T cell antigen OX40 [Mallet et al., EMBO J., 9:1063  
(1990)] and the Fas antigen [Yonehara et al., supra and Itoh et al.,  
supra]. CRDs are also found in the soluble TNFR (sTNFR)-like T2  
25 proteins of the Shope and myxoma poxviruses [Upton et al., Virology,  
160:20-29 (1987); Smith et al., Biochem. Biophys. Res. Commun., 176:335  
(1991); Upton et al., Virology, 184:370 (1991)]. Optimal alignment of  
these sequences indicates that the positions of the cysteine residues  
are well conserved. These receptors are sometimes collectively  
30 referred to as members of the TNF/NGF receptor superfamily. Recent  
studies on p75NGFR showed that the deletion of CRD1 [Welcher, A.A. et  
al., Proc. Natl. Acad. Sci. USA, 88:159-163 (1991)] or a 5-amino acid  
insertion in this domain [Yan, H. and Chao, M.V., J. Biol. Chem.,  
266:12099-12104 (1991)] had little or no effect on NGF binding [Yan, H.  
and Chao, M.V., supra]. p75 NGFR contains a proline-rich stretch of  
35 about 60 amino acids, between its CRD4 and transmembrane region, which  
is not involved in NGF binding [Peetre, C. et al., Eur. J. Hematol.,  
41:414-419 (1988); Seckinger, P. et al., J. Biol. Chem., 264:11966-11973  
(1989); Yan, H. and Chao, M.V., supra]. A similar proline-rich region  
is found in TNFR2 but not in TNFR1.

40 Itoh et al. disclose that the Apo-1 receptor can signal an  
apoptotic cell death similar to that signaled by the 55-kDa TNFR1 [Itoh  
et al., supra]. Expression of the Apo-1 antigen has also been reported



5 to be down-regulated along with that of TNFR1 when cells are treated  
with either TNF- $\alpha$  or anti-Apo-1 mouse monoclonal antibody [Krammer et  
al., supra; Nagata et al., supra]. Accordingly, some investigators have  
hypothesized that cell lines that co-express both Apo-1 and TNFR1  
10 [Id.] .

The TNF family ligands identified to date, with the exception  
of lymphotoxin- $\alpha$ , are type II transmembrane proteins, whose C-terminus  
is extracellular. In contrast, the receptors in the TNF receptor (TNFR)  
family identified to date are type I transmembrane proteins. In both  
15 the TNF ligand and receptor families, however, homology identified  
between family members has been found mainly in the extracellular domain  
("ECD"). Several of the TNF family cytokines, including TNF- $\alpha$ , Apo-1  
ligand and CD40 ligand, are cleaved proteolytically at the cell surface;  
the resulting protein in each case typically forms a homotrimeric  
20 molecule that functions as a soluble cytokine. TNF receptor family  
proteins are also usually cleaved proteolytically to release soluble  
receptor ECDs that can function as inhibitors of the cognate cytokines.

Recently, other members of the TNFR family have been  
identified. In Marsters et al., Curr. Biol., 6:750 (1996),  
25 investigators describe a full length native sequence human polypeptide,  
called Apo-3, which exhibits similarity to the TNFR family in its  
extracellular cysteine-rich repeats and resembles TNFR1 and CD95 in that  
it contains a cytoplasmic death domain sequence [see also Marsters et  
al., Curr. Biol., 6:1669 (1996)]. Apo-3 has also been referred to by  
30 other investigators as DR3, wsl-1 and TRAMP [Chinnaiyan et al., Science,  
274:990 (1996); Kitson et al., Nature, 384:372 (1996); Bodmer et al.,  
Immunity, 6:79 (1997)].

Pan et al. have disclosed another TNF receptor family member  
referred to as "DR4" [Pan et al., Science, 276:111-113 (1997)]. The DR4  
35 was reported to contain a cytoplasmic death domain capable of engaging  
the cell suicide apparatus. Pan et al. disclose that DR4 is believed to  
be a receptor for the ligand known as Apo-2 ligand or TRAIL.

#### The Apoptosis-Inducing Signaling Complex

As presently understood, the cell death program contains at  
40 least three important elements - activators, inhibitors, and effectors;  
in *C. elegans*, these elements are encoded respectively by three genes,

5 *Ced-4*, *Ced-9* and *Ced-3* [Steller, Science, 267:1445 (1995); Chinnaiyan et al., Science, 275:1122-1126 (1997)]. Two of the TNFR family members, TNFR1 and Fas/Apo1 (CD95), can activate apoptotic cell death [Chinnaiyan and Dixit, Current Biology, 6:555-562 (1996); Fraser and Evan, Cell; 85:781-784 (1996)]. TNFR1 is also known to mediate activation of the

10 transcription factor, NF-KB [Tartaglia et al., Cell, 74:845-853 (1993); Hsu et al., Cell, 84:299-308 (1996)]. In addition to some ECD homology, these two receptors share homology in their intracellular domain (ICD) in an oligomerization interface known as the death domain [Tartaglia et al., supra; Nagata, Cell, 88:355 (1997)]. Death domains are also found

15 in several metazoan proteins that regulate apoptosis, namely, the *Drosophila* protein, Reaper, and the mammalian proteins referred to as FADD/MORT1, TRADD, and RIP [Cleaveland and Ihle, Cell, 81:479-482 (1995)]. Using the yeast-two hybrid system, Raven et al. report the identification of protein, wsl-1, which binds to the TNFR1 death domain

20 [Raven et al., Programmed Cell Death Meeting, September 20-24, 1995, Abstract at page 127; Raven et al., European Cytokine Network, 7:Abstr. 82 at page 210 (April-June 1996)]. The wsl-1 protein is described as being homologous to TNFR1 (48% identity) and having a restricted tissue distribution. According to Raven et al., the tissue distribution of

25 wsl-1 is significantly different from the TNFR1 binding protein, TRADD.

Upon ligand binding and receptor clustering, TNFR1 and CD95 are believed to recruit FADD into a death-inducing signalling complex. CD95 purportedly binds FADD directly, while TNFR1 binds FADD indirectly via TRADD [Chinnaiyan et al., Cell, 81:505-512 (1995); Boldin et al., J. Biol. Chem., 270:387-391 (1995); Hsu et al., supra; Chinnaiyan et al., J. Biol. Chem., 271:4961-4965 (1996)]. It has been reported that FADD serves as an adaptor protein which recruits the *Ced-3*-related protease, MACH $\alpha$ /FLICE (caspase 8), into the death signalling complex [Boldin et al., Cell, 85:803-815 (1996); Muzio et al., Cell, 85:817-827 (1996)].

35 MACH $\alpha$ /FLICE appears to be the trigger that sets off a cascade of apoptotic proteases, including the interleukin-1 $\beta$  converting enzyme (ICE) and CPP32/Yama, which may execute some critical aspects of the cell death programme [Fraser and Evan, supra].

It was recently disclosed that programmed cell death involves

40 the activity of members of a family of cysteine proteases related to the *C. elegans* cell death gene, *ced-3*, and to the mammalian IL-1-converting

5 enzyme, ICE. The activity of the ICE and CPP32/Yama proteases can be inhibited by the product of the cowpox virus gene, *crmA* [Ray et al., Cell, 69:597-604 (1992); Tewari et al., Cell, 81:801-809 (1995)]. Recent studies show that CrmA can inhibit TNFR1- and CD95-induced cell death [Enari et al., Nature, 375:78-81 (1995); Tewari et al., J. Biol. Chem., 270:3255-3260 (1995)].

10 As reviewed recently by Tewari et al., TNFR1, TNFR2 and CD40 modulate the expression of proinflammatory and costimulatory cytokines, cytokine receptors, and cell adhesion molecules through activation of the transcription factor, NF-KB [Tewari et al., Curr. Op. Genet. Develop., 6:39-44 (1996)]. NF-KB is the prototype of a family of dimeric transcription factors whose subunits contain conserved Rel regions [Verma et al., Genes Develop., 9:2723-2735 (1996); Baldwin, Ann. Rev. Immunol., 14:649-681 (1996)]. In its latent form, NF-KB is complexed with members of the IKB inhibitor family; upon inactivation of the IKB in response to certain stimuli, released NF-KB translocates to the nucleus where it binds to specific DNA sequences and activates gene transcription.

20 For a review of the TNF family of cytokines and their receptors, see Gruss and Dower, supra.

#### 25 SUMMARY OF THE INVENTION

Applicants have identified cDNA clones that encode novel polypeptides, designated in the present application as "Apo-2." It is believed that Apo-2 is a member of the TNFR family; full-length native sequence human Apo-2 polypeptide exhibits some similarities to some known TNFRs, including a cytoplasmic death domain region. Full-length native sequence human Apo-2 also exhibits similarity to the TNFR family in its extracellular cysteine-rich repeats. Apo-2 polypeptide has been found to be capable of triggering caspase-dependent apoptosis and activating NF-KB. Applicants surprisingly found that the soluble extracellular domain of Apo-2 binds Apo-2 ligand ("Apo-2L") and can inhibit Apo-2 ligand function. It is presently believed that Apo-2 ligand can signal via at least two different receptors, DR4 and the newly described Apo-2 herein.

40 In one embodiment, the invention provides isolated Apo-2

polypeptide. In particular, the invention provides isolated native sequence Apo-2 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). In other embodiments, the isolated Apo-2 polypeptide comprises at least about 80% amino acid sequence identity with native sequence Apo-2 polypeptide comprising residues 1 to 411 of Figure 1 (SEQ ID NO:1). Optionally, the Apo-2 polypeptide is obtained or obtainable by expressing the polypeptide encoded by the cDNA insert of the vector deposited as ATCC 209021.

In another embodiment, the invention provides an isolated extracellular domain (ECD) sequence of Apo-2. Optionally, the isolated extracellular domain sequence comprises amino acid residues 54 to 182 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides an isolated death domain sequence of Apo-2. Optionally, the isolated death domain sequence comprises amino acid residues 324 to 391 of Fig. 1 (SEQ ID NO:1).

In another embodiment, the invention provides chimeric molecules comprising Apo-2 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises an Apo-2 fused to an immunoglobulin sequence. Another example comprises an extracellular domain sequence of Apo-2 fused to a heterologous polypeptide or amino acid sequence, such as an immunoglobulin sequence.

In another embodiment, the invention provides an isolated nucleic acid molecule encoding Apo-2 polypeptide. In one aspect, the nucleic acid molecule is RNA or DNA that encodes an Apo-2 polypeptide or a particular domain of Apo-2, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In one embodiment, the nucleic acid sequence is selected from:

- (a) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 411 (i.e., nucleotides 140-142 through 1370-1372), inclusive;
- (b) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 1 to residue 182 (i.e., nucleotides 140-142 through 683-685), inclusive;
- (c) the coding region of the nucleic acid sequence of Figure

5 1 (SEQ ID NO:2) that codes for residue 54 to residue 182 (i.e., nucleotides 299-301 through 683-685), inclusive;

(d) the coding region of the nucleic acid sequence of Figure 1 (SEQ ID NO:2) that codes for residue 324 to residue 391 (i.e., nucleotides 1109-1111 through 1310-1312), inclusive; or

10 (e) a sequence corresponding to the sequence of (a), (b), (c) or (d) within the scope of degeneracy of the genetic code. The isolated nucleic acid may comprise the Apo-2 polypeptide cDNA insert of the vector deposited as ATCC 209021 which includes the nucleotide sequence encoding Apo-2 polypeptide.

15 In a further embodiment, the invention provides a vector comprising the nucleic acid molecule encoding the Apo-2 polypeptide or particular domain of Apo-2. A host cell comprising the vector or the nucleic acid molecule is also provided. A method of producing Apo-2 is further provided.

20 In another embodiment, the invention provides an antibody which specifically binds to Apo-2. The antibody may be an agonistic, antagonistic or neutralizing antibody. Dimeric molecules, in particular homodimeric molecules, comprising Apo-2 antibody are also provided.

25 In another embodiment, the invention provides non-human, transgenic or knock-out animals.

A further embodiment of the invention provides articles of manufacture and kits that include Apo-2 or Apo-2 antibodies.

#### BRIEF DESCRIPTION OF THE DRAWINGS

30 Figure 1 shows the nucleotide sequence of a native sequence human Apo-2 cDNA and its derived amino acid sequence.

35 Figure 2A shows the derived amino acid sequence of a native sequence human Apo-2 - the putative signal sequence is underlined, the putative transmembrane domain is boxed, and the putative death domain sequence is dash underlined. The cysteines of the two cysteine-rich domains are individually underlined.

40 Figure 2B shows an alignment and comparison of the death domain sequences of native sequence human Apo-2, DR4, Apo-3/DR3, TNFR1, and Fas/Apo-1 (CD95). Asterisks indicate residues that are essential for death signaling by TNFR1 [Tartaglia et al., supra].

Figure 3 shows the interaction of the Apo-2 ECD with Apo-2L. Supernatants from mock-transfected 293 cells or from 293 cells

5 transfected with Flag epitope-tagged Apo-2 ECD were incubated with poly-His-tagged Apo-2L and subjected to immunoprecipitation with anti-Flag conjugated or Nickel conjugated agarose beads. The precipitated proteins were resolved by electrophoresis on polyacrylamide gels, and detected by immunoblot with anti-Apo-2L or anti-Flag antibody.

10 Figure 4 shows the induction of apoptosis by Apo-2 and inhibition of Apo-2L activity by soluble Apo-2 ECD. Human 293 cells (A, B) or HeLa cells (C) were transfected by pRK5 vector or by pRK5-based plasmids encoding Apo-2 and/or CrmA. Apoptosis was assessed by morphology (A), DNA fragmentation (B), or by FACS (C-E). Soluble Apo-2L  
15 was pre-incubated with buffer or affinity-purified Apo-2 ECD together with anti-Flag antibody or Apo-2 ECD immunoadhesin or DR4 or TNFR1 immunoadhesins and added to HeLa cells. The cells were later analyzed for apoptosis (D). Dose-response analysis using Apo-2L with Apo-2 ECD immunoadhesin was also determined (E).

20 Figure 5 shows activation of NF-KB by Apo-2, DR4, and Apo-2L. (A) HeLa cells were transfected with expression plasmids encoding the indicated proteins. Nuclear extracts were prepared and analyzed by an electrophoretic mobility shift assay. (B) HeLa cells or MCF7 cells were treated with buffer, Apo-2L or TNF-alpha and assayed for NF-KB  
25 activity. (C) HeLa cells were preincubated with buffer, ALLN or cyclohexamide before addition of Apo-2L. Apoptosis was later analyzed by FACS.

Figure 6 shows expression of Apo-2 mRNA in human tissues as analyzed by Northern hybridization of human tissue poly A RNA blots.

30 Figure 7 shows the FACS analysis of an Apo-2 antibody, 3F11.39.7 (illustrated by the bold lines) as compared to IgG controls (dotted lines). The 3F11.39.7 antibody recognized the Apo-2 receptor expressed in human 9D cells.

35 Figure 8 is a graph showing percent (%) apoptosis induced in 9D cells by Apo-2 antibody 3F11.39.7, in the absence of goat anti-mouse IgG Fc.

Figure 9 is a bar diagram showing percent (%) apoptosis, as compared to Apo-2L, in 9D cells by Apo-2 antibody 3F11.39.7 in the presence or absence of goat anti-mouse IgG Fc.

40 Figure 10 is a bar diagram illustrating the ability of Apo-2 antibody 3F11.39.7 to block the apoptosis induced by Apo-2L in 9D cells.

5 Figure 11 is a graph showing results of an ELISA testing binding of Apo-2 antibody 3F11.39.7 to Apo-2 and to other known Apo-2L receptors referred to as DR4, DcR1, and DcR2.

10 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "Apo-2 polypeptide" and "Apo-2" when used herein encompass native sequence Apo-2 and Apo-2 variants (which are further defined herein). These terms encompass Apo-2 from a variety of mammals, including humans. The Apo-2 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence Apo-2" comprises a polypeptide having the same amino acid sequence as an Apo-2 derived from nature. Thus, a native sequence Apo-2 can have the amino acid sequence of naturally-occurring Apo-2 from any mammal. Such native sequence Apo-2 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence Apo-2" specifically encompasses naturally-occurring truncated or secreted forms of the Apo-2 (e.g., an extracellular domain sequence), naturally-occurring variant forms (e.g., alternatively spliced forms) and naturally-occurring allelic variants of the Apo-2. A naturally-occurring variant form of the Apo-2 includes an Apo-2 having an amino acid substitution at residue 410 in the amino acid sequence shown in Figure 1 (SEQ ID NO:1). In one embodiment of such naturally-occurring variant form, the leucine residue at position 410 is substituted by a methionine residue. In Fig. 1 (SEQ ID NO:1), the amino acid residue at position 410 is identified as "Xaa" to indicate that the amino acid may, optionally, be either leucine or methionine. In Fig. 1 (SEQ ID NO:2), the nucleotide at position 1367 is identified as "W" to indicate that the nucleotide may be either adenine (A) or thymine (T) or uracil (U). In one embodiment of the invention, the native sequence Apo-2 is a mature or full-length native sequence Apo-2 comprising amino acids 1 to 411 of Fig. 1 (SEQ ID NO:1).

The "Apo-2 extracellular domain" or "Apo-2 ECD" refers to a form of Apo-2 which is essentially free of the transmembrane and cytoplasmic domains of Apo-2. Ordinarily, Apo-2 ECD will have less than 1% of such transmembrane and/or cytoplasmic domains and preferably, will





disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the Apo-2 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" Apo-2 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the Apo-2 nucleic acid. An isolated Apo-2 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated Apo-2 nucleic acid molecules therefore are distinguished from the Apo-2 nucleic acid molecule as it exists in natural cells. However, an isolated Apo-2 nucleic acid molecule includes Apo-2 nucleic acid molecules contained in cells that ordinarily express Apo-2 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding

5 sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If  
10 such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers anti-Apo-2 monoclonal antibodies (including agonist, antagonist, and blocking or neutralizing antibodies) and anti-Apo-2  
15 antibody compositions with polyepitopic specificity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may  
20 be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a  
25 single determinant on the antigen.

The monoclonal antibodies herein include hybrid and recombinant antibodies produced by splicing a variable (including hypervariable) domain of an anti-Apo-2 antibody with a constant domain, or a light chain with a heavy chain, or a chain from one species with a  
30 chain from another species, or fusions with heterologous proteins, regardless of species of origin or immunoglobulin class or subclass designation, as well as antibody fragments (e.g., Fab, F(ab')<sub>2</sub>, and Fv), so long as they exhibit the desired biological activity. See, e.g. U.S. Pat. No. 4,816,567 and Mage et al., in Monoclonal Antibody Production  
35 Techniques and Applications, pp.79-97 (Marcel Dekker, Inc.: New York, 1987).

Thus, the modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring  
40 production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler

5 and Milstein, Nature, 256:495 (1975), or may be made by recombinant DNA methods such as described in U.S. Pat. No. 4,816,567. The "monoclonal antibodies" may also be isolated from phage libraries generated using the techniques described in McCafferty et al., Nature, 348:552-554 (1990), for example.

10 "Humanized" forms of non-human (e.g. murine) antibodies are specific chimeric immunoglobulins, immunoglobulin chains, or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are  
15 human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat, or rabbit having the desired specificity, affinity, and capacity. In some instances, Fv framework region (FR) residues of the  
20 human immunoglobulin are replaced by corresponding non-human residues. Furthermore, the humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will  
25 comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion  
30 of an immunoglobulin constant region or domain (Fc), typically that of a human immunoglobulin.

"Biologically active" and "desired biological activity" for the purposes herein mean having the ability to modulate apoptosis (either in an agonistic or stimulating manner or in an antagonistic or  
35 blocking manner) in at least one type of mammalian cell *in vivo* or *ex vivo*.

The terms "apoptosis" and "apoptotic activity" are used in a broad sense and refer to the orderly or controlled form of cell death in mammals that is typically accompanied by one or more characteristic cell  
40 changes, including condensation of cytoplasm, loss of plasma membrane microvilli, segmentation of the nucleus, degradation of chromosomal DNA or loss of mitochondrial function. This activity can be determined and

5 measured, for instance, by cell viability assays, FACS analysis or DNA electrophoresis, all of which are known in the art.

The terms "treating," "treatment," and "therapy" as used herein refer to curative therapy, prophylactic therapy, and preventative therapy.

10 The term "mammal" as used herein refers to any mammal classified as a mammal, including humans, cows, horses, dogs and cats. In a preferred embodiment of the invention, the mammal is a human.

## 15 II. Compositions and Methods of the Invention

The present invention provides newly identified and isolated Apo-2 polypeptides and Apo-2 antibodies. In particular, Applicants have identified and isolated various human Apo-2 polypeptides. The properties and characteristics of some of these Apo-2 polypeptides and anti-Apo-2 antibodies are described in further detail in the Examples below. Based upon the properties and characteristics of the Apo-2 polypeptides disclosed herein, it is Applicants' present belief that Apo-2 is a member of the TNFR family.

20 A description follows as to how Apo-2, as well as Apo-2 chimeric molecules and anti-Apo-2 antibodies, may be prepared.

### 25 A. Preparation of Apo-2

The description below relates primarily to production of Apo-2 by culturing cells transformed or transfected with a vector containing Apo-2 nucleic acid. It is of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare Apo-2.

#### 30 1. Isolation of DNA Encoding Apo-2

The DNA encoding Apo-2 may be obtained from any cDNA library prepared from tissue believed to possess the Apo-2 mRNA and to express it at a detectable level. Accordingly, human Apo-2 DNA can be conveniently obtained from a cDNA library prepared from human tissues, such as the bacteriophage libraries of human pancreas and kidney cDNA described in Example 1. The Apo-2-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

40 Libraries can be screened with probes (such as antibodies to the Apo-2 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening

5 the cDNA or genomic library with the selected probe may be conducted  
using standard procedures, such as described in Sambrook et al.,  
Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor  
Laboratory Press, 1989). An alternative means to isolate the gene  
encoding Apo-2 is to use PCR methodology [Sambrook et al., supra;  
10 Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor  
Laboratory Press, 1995)].

A preferred method of screening employs selected  
oligonucleotide sequences to screen cDNA libraries from various human  
tissues. Example 1 below describes techniques for screening a cDNA  
15 library. The oligonucleotide sequences selected as probes should be of  
sufficient length and sufficiently unambiguous that false positives are  
minimized. The oligonucleotide is preferably labeled such that it can  
be detected upon hybridization to DNA in the library being screened.  
Methods of labeling are well known in the art, and include the use of  
20 radiolabels like <sup>32</sup>P-labeled ATP, biotinylation or enzyme labeling.  
Hybridization conditions, including moderate stringency and high  
stringency, are provided in Sambrook et al., supra.

Nucleic acid having all the protein coding sequence may be  
obtained by screening selected cDNA or genomic libraries using the  
deduced amino acid sequence disclosed herein for the first time, and, if  
25 necessary, using conventional primer extension procedures as described  
in Sambrook et al., supra, to detect precursors and processing  
intermediates of mRNA that may not have been reverse-transcribed into  
cDNA.

Apo-2 variants can be prepared by introducing appropriate  
nucleotide changes into the Apo-2 DNA, or by synthesis of the desired  
Apo-2 polypeptide. Those skilled in the art will appreciate that amino  
acid changes may alter post-translational processes of the Apo-2, such  
as changing the number or position of glycosylation sites or altering  
35 the membrane anchoring characteristics.

Variations in the native full-length sequence Apo-2 or in  
various domains of the Apo-2 described herein, can be made, for example,  
using any of the techniques and guidelines for conservative and non-  
conservative mutations set forth, for instance, in U.S. Pat. No.  
40 5,364,934. Variations may be a substitution, deletion or insertion of  
one or more codons encoding the Apo-2 that results in a change in the  
amino acid sequence of the Apo-2 as compared with the native sequence

5 Apo-2. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the Apo-2 molecule. The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis  
10 [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the Apo-2 variant DNA.

15 Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence which are involved in the interaction with a particular ligand or receptor. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine.  
20 Alanine is the preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

Once selected Apo-2 variants are produced, they can be contacted with, for instance, Apo-2L, and the interaction, if any, can  
30 be determined. The interaction between the Apo-2 variant and Apo-2L can be measured by an *in vitro* assay, such as described in the Examples below. While any number of analytical measurements can be used to compare activities and properties between a native sequence Apo-2 and an Apo-2 variant, a convenient one for binding is the dissociation constant  
35  $K_d$  of the complex formed between the Apo-2 variant and Apo-2L as compared to the  $K_d$  for the native sequence Apo-2. Generally, a  $\geq 3$ -fold increase or decrease in  $K_d$  per substituted residue indicates that the substituted residue(s) is active in the interaction of the native sequence Apo-2 with the Apo-2L.

40 Optionally, representative sites in the Apo-2 sequence suitable for mutagenesis would include sites within the extracellular domain, and particularly, within one or both of the cysteine-rich

5 domains. Such variations can be accomplished using the methods described above.

## 2. Insertion of Nucleic Acid into A Replicable Vector

10 The nucleic acid (e.g., cDNA or genomic DNA) encoding Apo-2 may be inserted into a replicable vector for further cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector components generally include, but are not limited to, one or more of the following: a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence, each of which is  
15 described below.

### (i) Signal Sequence Component

20 The Apo-2 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the Apo-2 DNA that is inserted into the vector. The heterologous signal sequence selected preferably is one that is recognized and processed (i.e., cleaved by a signal peptidase) by the  
25 host cell. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, e.g., the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor  
30 leaders, the latter described in U.S. Pat. No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression the native Apo-2 presequence that normally directs insertion of Apo-2 in the cell  
35 membrane of human cells *in vivo* is satisfactory, although other mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex glycoprotein D signal.

40 The DNA for such precursor region is preferably ligated in reading frame to DNA encoding Apo-2.

5 (ii) Origin of Replication Component

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Generally, in cloning vectors this sequence is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 $\mu$  plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, the origin of replication component is not needed for mammalian expression vectors (the SV40 origin may typically be used because it contains the early promoter).

Most expression vectors are "shuttle" vectors, i.e., they are capable of replication in at least one class of organisms but can be transfected into another organism for expression. For example, a vector is cloned in *E. coli* and then the same vector is transfected into yeast or mammalian cells for expression even though it is not capable of replicating independently of the host cell chromosome.

DNA may also be amplified by insertion into the host genome. This is readily accomplished using *Bacillus* species as hosts, for example, by including in the vector a DNA sequence that is complementary to a sequence found in *Bacillus* genomic DNA. Transfection of *Bacillus* with this vector results in homologous recombination with the genome and insertion of Apo-2 DNA. However, the recovery of genomic DNA encoding Apo-2 is more complex than that of an exogenously replicated vector because restriction enzyme digestion is required to excise the Apo-2 DNA.

(iii) Selection Gene Component

Expression and cloning vectors typically contain a selection gene, also termed a selectable marker. This gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate,



5 or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for *Bacilli*.

10 One example of a selection scheme utilizes a drug to arrest growth of a host cell. Those cells that are successfully transformed with a heterologous gene produce a protein conferring drug resistance and thus survive the selection regimen. Examples of such dominant selection use the drugs neomycin [Southern et al., J. Molec. Appl. Genet., 1:327 (1982)], mycophenolic acid (Mulligan et al., Science, 209:1422 (1980)] or hygromycin [Sugden et al., Mol. Cell. Biol., 5:410-413 (1985)]. The three examples given above employ bacterial genes under eukaryotic control to convey resistance to the appropriate drug G418 or neomycin (geneticin), xgpt (mycophenolic acid), or hygromycin, respectively.

20 Another example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the Apo-2 nucleic acid, such as DHFR or thymidine kinase. The mammalian cell transformants are placed under selection pressure that only the transformants are uniquely adapted to survive by virtue of having taken up the marker. Selection pressure is imposed by culturing the transformants under conditions in which the concentration of selection agent in the medium is successively changed, thereby leading to amplification of both the selection gene and the DNA that encodes Apo-2. Amplification is the process by which genes in greater demand for the production of a protein critical for growth are reiterated in tandem within the chromosomes of successive generations of recombinant cells. Increased quantities of Apo-2 are synthesized from the amplified DNA. Other examples of amplifiable genes include metallothionein-I and -II, adenosine deaminase, and ornithine decarboxylase.

35 Cells transformed with the DHFR selection gene may first be identified by culturing all of the transformants in a culture medium that contains methotrexate (Mtx), a competitive antagonist of DHFR. An appropriate host cell when wild-type DHFR is employed is the Chinese hamster ovary (CHO) cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). The transformed cells are then exposed to increased levels of methotrexate. This leads to the synthesis of multiple copies of the DHFR gene, and, concomitantly, multiple copies of other DNA

5 comprising the expression vectors, such as the DNA encoding Apo-2. This amplification technique can be used with any otherwise suitable host, e.g., ATCC No. CCL61 CHO-K1, notwithstanding the presence of endogenous DHFR if, for example, a mutant DHFR gene that is highly resistant to Mtx is employed (EP 117,060).

10 Alternatively, host cells (particularly wild-type hosts that contain endogenous DHFR) transformed or co-transformed with DNA sequences encoding Apo-2, wild-type DHFR protein, and another selectable marker such as aminoglycoside 3'-phosphotransferase (APH) can be selected by cell growth in medium containing a selection agent for the  
15 selectable marker such as an aminoglycosidic antibiotic, e.g., kanamycin, neomycin, or G418. See U.S. Patent No. 4,965,199.

A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 [Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene,  
20 10:157 (1980)]. The *trp1* gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, Genetics, 85:12 (1977)]. The presence of the *trp1* lesion in the yeast host cell genome then provides an effective environment for detecting transformation by growth in the  
25 absence of tryptophan. Similarly, *Leu2*-deficient yeast strains (ATCC 20,622 or 38,626) are complemented by known plasmids bearing the *Leu2* gene.

In addition, vectors derived from the 1.6  $\mu$ m circular plasmid pKD1 can be used for transformation of *Kluyveromyces* yeasts [Bianchi et  
30 al., Curr. Genet., 12:185 (1987)]. More recently, an expression system for large-scale production of recombinant calf chymosin was reported for *K. lactis* [Van den Berg, Bio/Technology, 8:135 (1990)]. Stable multi-copy expression vectors for secretion of mature recombinant human serum albumin by industrial strains of *Kluyveromyces* have also been disclosed  
35 [Fleer et al., Bio/Technology, 9:968-975 (1991)].

#### (iv) Promoter Component

Expression and cloning vectors usually contain a promoter that is recognized by the host organism and is operably linked to the Apo-2 nucleic acid sequence. Promoters are untranslated sequences  
40 located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and

translation of particular nucleic acid sequence, such as the Apo-2 nucleic acid sequence, to which they are operably linked. Such promoters typically fall into two classes, inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature. At this time a large number of promoters recognized by a variety of potential host cells are well known. These promoters are operably linked to Apo-2 encoding DNA by removing the promoter from the source DNA by restriction enzyme digestion and inserting the isolated promoter sequence into the vector. Both the native Apo-2 promoter sequence and many heterologous promoters may be used to direct amplification and/or expression of the Apo-2 DNA.

Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems [Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)]. However, other known bacterial promoters are suitable. Their nucleotide sequences have been published, thereby enabling a skilled worker operably to ligate them to DNA encoding Apo-2 [Siebenlist et al., Cell, 20:269 (1980)] using linkers or adaptors to supply any required restriction sites. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding Apo-2.

Promoter sequences are known for eukaryotes. Virtually all eukaryotic genes have an AT-rich region located approximately 25 to 30 bases upstream from the site where transcription is initiated. Another sequence found 70 to 80 bases upstream from the start of transcription of many genes is a CXCAAT region where X may be any nucleotide. At the 3' end of most eukaryotic genes is an AATAAA sequence that may be the signal for addition of the poly A tail to the 3' end of the coding sequence. All of these sequences are suitably inserted into eukaryotic expression vectors.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman et

5 al., J. Biol. Chem., 255:2073 (1980)] or other glycolytic enzymes [Hess  
et al., J. Adv. Enzyme Req., 7:149 (1968); Holland, Biochemistry,  
17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate  
dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase,  
10 kinase, triosephosphate isomerase, phosphoglucose isomerase, and  
glucokinase.

Other yeast promoters, which are inducible promoters having  
the additional advantage of transcription controlled by growth  
conditions, are the promoter regions for alcohol dehydrogenase 2,  
15 isocytochrome C, acid phosphatase, degradative enzymes associated with  
nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate  
dehydrogenase, and enzymes responsible for maltose and galactose  
utilization. Suitable vectors and promoters for use in yeast expression  
are further described in EP 73,657. Yeast enhancers also are  
20 advantageously used with yeast promoters.

Apo-2 transcription from vectors in mammalian host cells is  
controlled, for example, by promoters obtained from the genomes of  
viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5  
July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus,  
25 avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus  
and most preferably Simian Virus 40 (SV40), from heterologous mammalian  
promoters, e.g., the actin promoter or an immunoglobulin promoter, from  
heat-shock promoters, and from the promoter normally associated with the  
Apo-2 sequence, provided such promoters are compatible with the host  
30 cell systems.

The early and late promoters of the SV40 virus are  
conveniently obtained as an SV40 restriction fragment that also contains  
the SV40 viral origin of replication [Fiers et al., Nature, 273:113  
(1978); Mulligan and Berg, Science, 209:1422-1427 (1980); Pavlakis et  
35 al., Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)]. The immediate  
early promoter of the human cytomegalovirus is conveniently obtained as  
a HindIII E restriction fragment [Greenaway et al., Gene, 18:355-360  
(1982)]. A system for expressing DNA in mammalian hosts using the bovine  
papilloma virus as a vector is disclosed in U.S. Patent No. 4,419,446.  
40 A modification of this system is described in U.S. Patent No. 4,601,978  
[See also Gray et al., Nature, 295:503-508 (1982) on expressing cDNA  
encoding immune interferon in monkey cells; Reyes et al., Nature,

5 297:598-601 (1982) on expression of human  $\beta$ -interferon cDNA in mouse  
cells under the control of a thymidine kinase promoter from herpes  
simplex virus; Canaani and Berg, Proc. Natl. Acad. Sci. USA 79:5166-5170  
(1982) on expression of the human interferon gene in cultured mouse and  
10 6781 (1982) on expression of bacterial CAT sequences in CV-1 monkey  
kidney cells, chicken embryo fibroblasts, Chinese hamster ovary cells,  
HeLa cells, and mouse NIH-3T3 cells using the Rous sarcoma virus long  
terminal repeat as a promoter].

(v) Enhancer Element Component

15 Transcription of a DNA encoding the Apo-2 of this invention  
by higher eukaryotes may be increased by inserting an enhancer sequence  
into the vector. Enhancers are cis-acting elements of DNA, usually  
about from 10 to 300 bp, that act on a promoter to increase its  
transcription. Enhancers are relatively orientation and position  
20 independent, having been found 5' [Laimins et al., Proc. Natl. Acad.  
Sci. USA, 78:993 (1981)] and 3' [Lusky et al., Mol. Cell Bio., 3:1108  
(1983)] to the transcription unit, within an intron [Banerji et al.,  
Cell, 33:729 (1983)], as well as within the coding sequence itself  
[Osborne et al., Mol. Cell Bio., 4:1293 (1984)]. Many enhancer  
25 sequences are now known from mammalian genes (globin, elastase, albumin,  
 $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an  
enhancer from a eukaryotic cell virus. Examples include the SV40  
enhancer on the late side of the replication origin (bp 100-270), the  
cytomegalovirus early promoter enhancer, the polyoma enhancer on the  
30 late side of the replication origin, and adenovirus enhancers. See also  
Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of  
eukaryotic promoters. The enhancer may be spliced into the vector at a  
position 5' or 3' to the Apo-2 coding sequence, but is preferably  
located at a site 5' from the promoter.

35 (vi) Transcription Termination Component

Expression vectors used in eukaryotic host cells (yeast,  
fungi, insect, plant, animal, human, or nucleated cells from other  
multicellular organisms) will also contain sequences necessary for the  
termination of transcription and for stabilizing the mRNA. Such  
40 sequences are commonly available from the 5' and, occasionally 3',  
untranslated regions of eukaryotic or viral DNAs or cDNAs. These

5 regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding Apo-2.

(vii) Construction and Analysis of Vectors

Construction of suitable vectors containing one or more of the above-listed components employs standard ligation techniques.

10 Isolated plasmids or DNA fragments are cleaved, tailored, and re-ligated in the form desired to generate the plasmids required.

For analysis to confirm correct sequences in plasmids constructed, the ligation mixtures can be used to transform *E. coli* K12 strain 294 (ATCC 31,446) and successful transformants selected by ampicillin or tetracycline resistance where appropriate. Plasmids from the transformants are prepared, analyzed by restriction endonuclease digestion, and/or sequenced by the method of Messing et al., Nucleic Acids Res., 9:309 (1981) or by the method of Maxim et al., Methods in Enzymology, 65:499 (1980).

20 (viii) Transient Expression Vectors

Expression vectors that provide for the transient expression in mammalian cells of DNA encoding Apo-2 may be employed. In general, transient expression involves the use of an expression vector that is able to replicate efficiently in a host cell, such that the host cell accumulates many copies of the expression vector and, in turn, synthesizes high levels of a desired polypeptide encoded by the expression vector [Sambrook et al., *supra*]. Transient expression systems, comprising a suitable expression vector and a host cell, allow for the convenient positive identification of polypeptides encoded by cloned DNAs, as well as for the rapid screening of such polypeptides for desired biological or physiological properties. Thus, transient expression systems are particularly useful in the invention for purposes of identifying Apo-2 variants.

(ix) Suitable Exemplary Vertebrate Cell Vectors

35 Other methods, vectors, and host cells suitable for adaptation to the synthesis of Apo-2 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

3. Selection and Transformation of Host Cells

40 Suitable host cells for cloning or expressing the DNA in the vectors herein are the prokaryote, yeast, or higher eukaryote cells described above. Suitable prokaryotes for this purpose include but are

5 not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, e.g., *E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, e.g., *Salmonella typhimurium*, *Serratia*, e.g., *Serratia marcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (e.g., *B. licheniformis* 41P disclosed in DD 266,710 published 12 April 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. Preferably, the host cell should secrete minimal amounts of proteolytic enzymes.

15 In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for Apo-2-encoding vectors. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein.

20 Suitable host cells for the expression of glycosylated Apo-2 are derived from multicellular organisms. Such host cells are capable of complex processing and glycosylation activities. In principle, any higher eukaryotic cell culture is workable, whether from vertebrate or invertebrate culture. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodoptera frugiperda* (caterpillar), *Aedes aegypti* (mosquito), *Aedes albopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyx mori* have been identified [See, e.g., Luckow et al., Bio/Technology, 6:47-55 (1988); Miller et al., in Genetic Engineering, Setlow et al., eds., Vol. 8 (Plenum Publishing, 1986), pp. 277-279; and Maeda et al., Nature, 315:592-594 (1985)]. A variety of viral strains for transfection are publicly available, e.g., the L-1 variant of *Autographa californica* NPV and the Bm-5 strain of *Bombyx mori* NPV.

35 Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, and tobacco can be utilized as hosts. Typically, plant cells are transfected by incubation with certain strains of the bacterium *Agrobacterium tumefaciens*. During incubation of the plant cell culture with *A. tumefaciens*, the DNA encoding the Apo-2 can be transferred to the plant cell host such that it is transfected, and will, under appropriate conditions, express the Apo-2-encoding DNA. In addition, regulatory and signal sequences compatible with plant cells are available, such as the nopaline synthase promoter and

5 polyadenylation signal sequences [Depicker et al., J. Mol. Appl. Gen.,  
1:561 (1982)]. In addition, DNA segments isolated from the upstream  
region of the T-DNA 780 gene are capable of activating or increasing  
transcription levels of plant-expressible genes in recombinant DNA-  
containing plant tissue [EP 321,196 published 21 June 1989].

10 Propagation of vertebrate cells in culture (tissue culture)  
is also well known in the art [See, e.g., Tissue Culture, Academic  
Press, Kruse and Patterson, editors (1973)]. Examples of useful  
mammalian host cell lines are monkey kidney CV1 line transformed by SV40  
(COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells  
15 subcloned for growth in suspension culture, Graham et al., J. Gen  
Virol., 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10);  
Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl.  
Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol.  
Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70);  
20 African green monkey kidney cells (VERO-76, ATCC CRL-1587); human  
cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK,  
ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human  
lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065);  
mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather et al.,  
25 Annals N.Y. Acad. Sci., 383:44-68 (1982)); MRC 5 cells; and FS4 cells.

Host cells are transfected and preferably transformed with  
the above-described expression or cloning vectors for Apo-2 production  
and cultured in conventional nutrient media modified as appropriate for  
inducing promoters, selecting transformants, or amplifying the genes  
30 encoding the desired sequences.

Transfection refers to the taking up of an expression vector  
by a host cell whether or not any coding sequences are in fact  
expressed. Numerous methods of transfection are known to the ordinarily  
skilled artisan, for example,  $\text{CaPO}_4$  and electroporation. Successful  
35 transfection is generally recognized when any indication of the  
operation of this vector occurs within the host cell.

Transformation means introducing DNA into an organism so that  
the DNA is replicable, either as an extrachromosomal element or by  
chromosomal integrant. Depending on the host cell used, transformation  
40 is done using standard techniques appropriate to such cells. The  
calcium treatment employing calcium chloride, as described in Sambrook  
et al., supra, or electroporation is generally used for prokaryotes or



5 other cells that contain substantial cell-wall barriers. Infection with  
*Agrobacterium tumefaciens* is used for transformation of certain plant  
cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859  
published 29 June 1989. In addition, plants may be transfected using  
ultrasound treatment as described in WO 91/00358 published 10 January  
10 1991.

For mammalian cells without such cell walls, the calcium  
phosphate precipitation method of Graham and van der Eb, Virology,  
52:456-457 (1978) is preferred. General aspects of mammalian cell host  
system transformations have been described in U.S. Pat. No. 4,399,216.  
15 Transformations into yeast are typically carried out according to the  
method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et  
al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other  
methods for introducing DNA into cells, such as by nuclear  
microinjection, electroporation, bacterial protoplast fusion with intact  
20 cells, or polycations, e.g., polybrene, polyornithine, may also be used.  
For various techniques for transforming mammalian cells, see Keown et  
al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al.,  
Nature, 336:348-352 (1988).

#### 4. Culturing the Host Cells

25 Prokaryotic cells used to produce Apo-2 may be cultured in  
suitable media as described generally in Sambrook et al., supra.

The mammalian host cells used to produce Apo-2 may be  
cultured in a variety of media. Examples of commercially available  
media include Ham's F10 (Sigma), Minimal Essential Medium ("MEM",  
30 Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium  
("DMEM", Sigma). Any such media may be supplemented as necessary with  
hormones and/or other growth factors (such as insulin, transferrin, or  
epidermal growth factor), salts (such as sodium chloride, calcium,  
magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as  
35 adenosine and thymidine), antibiotics (such as Gentamycin<sup>TM</sup> drug), trace  
elements (defined as inorganic compounds usually present at final  
concentrations in the micromolar range), and glucose or an equivalent  
energy source. Any other necessary supplements may also be included at  
appropriate concentrations that would be known to those skilled in the  
40 art. The culture conditions, such as temperature, pH, and the like, are  
those previously used with the host cell selected for expression, and  
will be apparent to the ordinarily skilled artisan.

5 In general, principles, protocols, and practical techniques for maximizing the productivity of mammalian cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991).

10 The host cells referred to in this disclosure encompass cells in culture as well as cells that are within a host animal.

#### 5. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis),  
15 or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Various labels may be employed, most commonly radioisotopes, and particularly <sup>32</sup>P. However, other techniques may also be employed, such as using biotin-modified nucleotides for  
20 introduction into a polynucleotide. The biotin then serves as the site for binding to avidin or antibodies, which may be labeled with a wide variety of labels, such as radionucleotides, fluorescers or enzymes. Alternatively, antibodies may be employed that can recognize specific  
25 duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by  
30 immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. With immunohistochemical staining techniques, a cell sample is prepared, typically by dehydration and fixation, followed by reaction with labeled antibodies specific for  
35 the gene product coupled, where the labels are usually visually detectable, such as enzymatic labels, fluorescent labels, or luminescent labels.

Antibodies useful for immunohistochemical staining and/or  
40 assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence Apo-2 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous

5 sequence fused to Apo-2 DNA and encoding a specific antibody epitope.

#### 6. Purification of Apo-2 Polypeptide

Forms of Apo-2 may be recovered from culture medium or from host cell lysates. If the Apo-2 is membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g. Triton-X  
10 100) or its extracellular domain may be released by enzymatic cleavage.

When Apo-2 is produced in a recombinant cell other than one of human origin, the Apo-2 is free of proteins or polypeptides of human origin. However, it may be desired to purify Apo-2 from recombinant cell proteins or polypeptides to obtain preparations that are  
15 substantially homogeneous as to Apo-2. As a first step, the culture medium or lysate may be centrifuged to remove particulate cell debris. Apo-2 thereafter is purified from contaminant soluble proteins and polypeptides, with the following procedures being exemplary of suitable purification procedures: by fractionation on an ion-exchange column;  
20 ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; and protein A Sepharose columns to remove contaminants such as IgG.

Apo-2 variants in which residues have been deleted, inserted, or substituted can be recovered in the same fashion as native sequence Apo-2, taking account of changes in properties occasioned by the variation. For example, preparation of an Apo-2 fusion with another  
25 protein or polypeptide, e.g., a bacterial or viral antigen, immunoglobulin sequence, or receptor sequence, may facilitate purification; an immunoaffinity column containing antibody to the  
30 sequence can be used to adsorb the fusion polypeptide. Other types of affinity matrices also can be used.

A protease inhibitor such as phenyl methyl sulfonyl fluoride (PMSF) also may be useful to inhibit proteolytic degradation during  
35 purification, and antibiotics may be included to prevent the growth of adventitious contaminants. One skilled in the art will appreciate that purification methods suitable for native sequence Apo-2 may require modification to account for changes in the character of Apo-2 or its  
40 variants upon expression in recombinant cell culture.

#### 7. Covalent Modifications of Apo-2 Polypeptides

Covalent modifications of Apo-2 are included within the scope

5 of this invention. One type of covalent modification of the Apo-2 is introduced into the molecule by reacting targeted amino acid residues of the Apo-2 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the Apo-2.

10 Derivatization with bifunctional agents is useful for crosslinking Apo-2 to a water-insoluble support matrix or surface for use in the method for purifying anti-Apo-2 antibodies, and vice-versa. Derivatization with one or more bifunctional agents will also be useful for crosslinking Apo-2 molecules to generate Apo-2 dimers. Such dimers  
15 may increase binding avidity and extend half-life of the molecule *in vivo*. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-  
20 (succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[(p-azidophenyl)dithio]propioimide yield photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen  
25 bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642; 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl  
30 residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)],  
35 acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group. The modified forms of the residues fall within the scope of the present invention.

Another type of covalent modification of the Apo-2 polypeptide included within the scope of this invention comprises  
40 altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to

5 mean deleting one or more carbohydrate moieties found in native sequence Apo-2, and/or adding one or more glycosylation sites that are not present in the native sequence Apo-2.

10 Glycosylation of polypeptides is typically either N-linked or O-linked. N-linked refers to the attachment of the carbohydrate moiety to the side chain of an asparagine residue. The tripeptide sequences asparagine-X-serine and asparagine-X-threonine, where X is any amino acid except proline, are the recognition sequences for enzymatic attachment of the carbohydrate moiety to the asparagine side chain. Thus, the presence of either of these tripeptide sequences in a  
15 polypeptide creates a potential glycosylation site. O-linked glycosylation refers to the attachment of one of the sugars N-acetylglactosamine, galactose, or xylose to a hydroxylamino acid, most commonly serine or threonine, although 5-hydroxyproline or 5-hydroxylysine may also be used.

20 Addition of glycosylation sites to the Apo-2 polypeptide may be accomplished by altering the amino acid sequence such that it contains one or more of the above-described tripeptide sequences (for N-linked glycosylation sites). The alteration may also be made by the addition of, or substitution by, one or more serine or threonine  
25 residues to the native sequence Apo-2 (for O-linked glycosylation sites). The Apo-2 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the Apo-2 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids. The DNA mutation(s)  
30 may be made using methods described above and in U.S. Pat. No. 5,364,934, supra.

Another means of increasing the number of carbohydrate moieties on the Apo-2 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Depending on the coupling mode used,  
35 the sugar(s) may be attached to (a) arginine and histidine, (b) free carboxyl groups, (c) free sulfhydryl groups such as those of cysteine, (d) free hydroxyl groups such as those of serine, threonine, or hydroxyproline, (e) aromatic residues such as those of phenylalanine, tyrosine, or tryptophan, or (f) the amide group of glutamine. These  
40 methods are described in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the Apo-2

5 polypeptide may be accomplished chemically or enzymatically or by  
mutational substitution of codons encoding for amino acid residues that  
serve as targets for glycosylation. For instance, chemical  
deglycosylation by exposing the polypeptide to the compound  
trifluoromethanesulfonic acid, or an equivalent compound can result in  
10 the cleavage of most or all sugars except the linking sugar (N-  
acetylglucosamine or N-acetylgalactosamine), while leaving the  
polypeptide intact. Chemical deglycosylation is described by  
Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge  
et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of  
15 carbohydrate moieties on polypeptides can be achieved by the use of a  
variety of endo- and exo-glycosidases as described by Thotakura et al.,  
Meth. Enzymol., 138:350 (1987).

Glycosylation at potential glycosylation sites may be  
prevented by the use of the compound tunicamycin as described by Duskin  
et al., J. Biol. Chem., 257:3105 (1982). Tunicamycin blocks the  
20 formation of protein-N-glycoside linkages.

Another type of covalent modification of Apo-2 comprises  
linking the Apo-2 polypeptide to one of a variety of nonproteinaceous  
polymers, e.g., polyethylene glycol, polypropylene glycol, or  
25 polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835;  
4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

#### 8. Apo-2 Chimeras

The present invention also provides chimeric molecules  
comprising Apo-2 fused to another, heterologous polypeptide or amino  
30 acid sequence.

In one embodiment, the chimeric molecule comprises a fusion  
of the Apo-2 with a tag polypeptide which provides an epitope to which  
an anti-tag antibody can selectively bind. The epitope tag is generally  
placed at the amino- or carboxyl- terminus of the Apo-2. The presence  
35 of such epitope-tagged forms of the Apo-2 can be detected using an  
antibody against the tag polypeptide. Also, provision of the epitope  
tag enables the Apo-2 to be readily purified by affinity purification  
using an anti-tag antibody or another type of affinity matrix that binds  
to the epitope tag.

40 Various tag polypeptides and their respective antibodies are  
well known in the art. Examples include the flu HA tag polypeptide and  
its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)];

5 the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto  
[Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and  
the Herpes Simplex virus glycoprotein D (gD) tag and its antibody  
[Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag  
10 polypeptides include the Flag-peptide [Hopp et al., BioTechnology,  
6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science,  
255:192-194 (1992)]; an  $\alpha$ -tubulin epitope peptide [Skinner et al., J.  
Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide  
tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397  
15 (1990)]. Once the tag polypeptide has been selected, an antibody  
thereto can be generated using the techniques disclosed herein.

Generally, epitope-tagged Apo-2 may be constructed and  
produced according to the methods described above. Epitope-tagged Apo-2  
is also described in the Examples below. Apo-2-tag polypeptide fusions  
are preferably constructed by fusing the cDNA sequence encoding the Apo-  
20 2 portion in-frame to the tag polypeptide DNA sequence and expressing  
the resultant DNA fusion construct in appropriate host cells.  
Ordinarily, when preparing the Apo-2-tag polypeptide chimeras of the  
present invention, nucleic acid encoding the Apo-2 will be fused at its  
3' end to nucleic acid encoding the N-terminus of the tag polypeptide,  
25 however 5' fusions are also possible. For example, a polyhistidine  
sequence of about 5 to about 10 histidine residues may be fused at the  
N- terminus or the C- terminus and used as a purification handle in  
affinity chromatography.

Epitope-tagged Apo-2 can be purified by affinity  
30 chromatography using the anti-tag antibody. The matrix to which the  
affinity antibody is attached may include, for instance, agarose,  
controlled pore glass or poly(styrenedivinyl)benzene. The epitope-  
tagged Apo-2 can then be eluted from the affinity column using  
techniques known in the art.

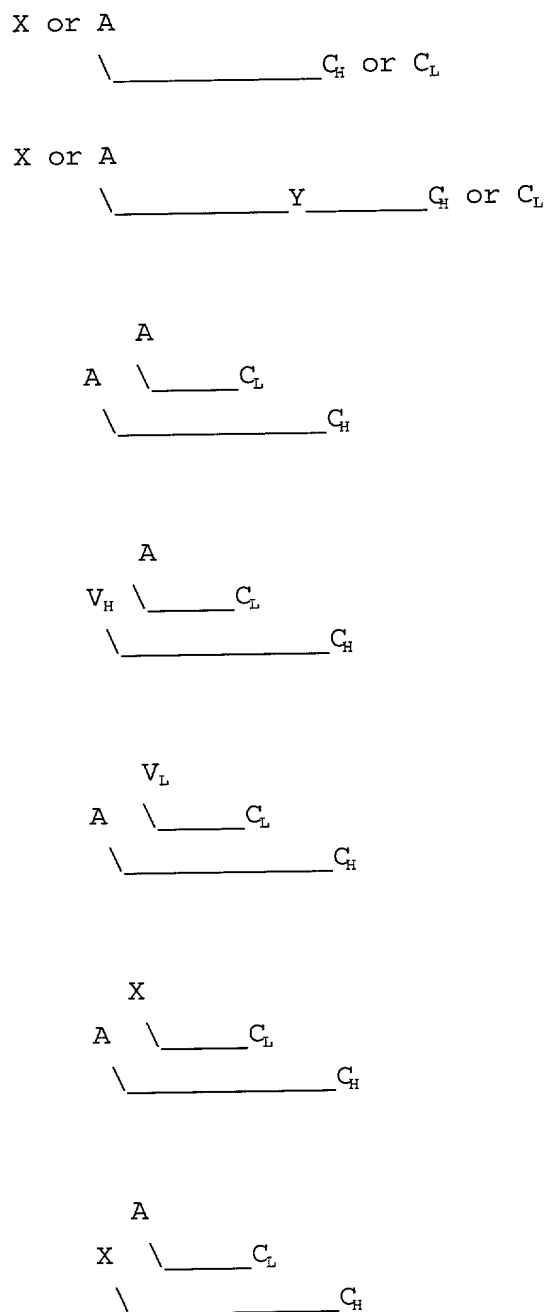
35 In another embodiment, the chimeric molecule comprises an  
Apo-2 polypeptide fused to an immunoglobulin sequence. The chimeric  
molecule may also comprise a particular domain sequence of Apo-2, such  
as the extracellular domain sequence of native Apo-2 fused to an  
immunoglobulin sequence. This includes chimeras in monomeric, homo- or  
40 heteromultimeric, and particularly homo- or heterodimeric, or -  
tetrameric forms; optionally, the chimeras may be in dimeric forms or

- 5 homodimeric heavy chain forms. Generally, these assembled immunoglobulins will have known unit structures as represented by the following diagrams.

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A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in



5

monomer: A \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>

10

homodimer: A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /  
 A

15

heterodimer: A  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 /

20

X

homotetramer:

25

A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 A /  
 A

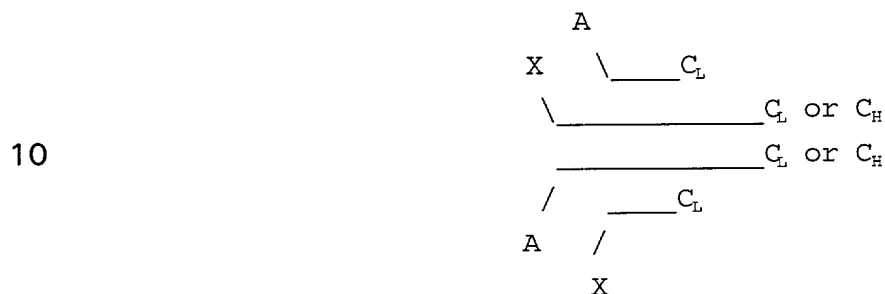
30

heterotetramer:

35

A  
 A \ \_\_\_\_\_ C<sub>L</sub>  
 \ \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 \_\_\_\_\_ C<sub>L</sub> or C<sub>H</sub>  
 / \_\_\_\_\_ C<sub>L</sub>  
 X /  
 X

5 and



15

20 In the foregoing diagrams, "A" means an Apo-2 sequence or an Apo-2 sequence fused to a heterologous sequence; X is an additional agent, which may be the same as A or different, a portion of an immunoglobulin superfamily member such as a variable region or a variable region-like domain, including a native or chimeric immunoglobulin variable region, a toxin such a pseudomonas exotoxin or ricin, or a sequence functionally binding to another protein, such as other cytokines (i.e., IL-1, interferon- $\gamma$ ) or cell surface molecules (i.e., NGFR, CD40, OX40, Fas antigen, T2 proteins of Shope and myxoma poxviruses), or a polypeptide therapeutic agent not otherwise normally associated with a constant domain; Y is a linker or another receptor sequence; and V<sub>L</sub>, V<sub>H</sub>, C<sub>L</sub> and C<sub>H</sub> represent light or heavy chain variable or constant domains of an immunoglobulin. Structures comprising at least one CRD of an Apo-2 sequence as "A" and another cell-surface protein having a repetitive pattern of CRDs (such as TNFR) as "X" are specifically included.

35 It will be understood that the above diagrams are merely exemplary of the possible structures of the chimeras of the present invention, and do not encompass all possibilities. For example, there might desirably be several different "A"s, "X"s, or "Y"s in any of these constructs. Also, the heavy or light chain constant domains may be originated from the same or different immunoglobulins. All possible permutations of the illustrated and similar structures are all within the scope of the invention herein.

40 In general, the chimeric molecules can be constructed in a fashion similar to chimeric antibodies in which a variable domain from

5 an antibody of one species is substituted for the variable domain of  
another species. See, for example, EP 0 125 023; EP 173,494; Munro,  
Nature, 312:597 (13 December 1984); Neuberger et al., Nature, 312:604-  
608 (13 December 1984); Sharon et al., Nature, 309:364-367 (24 May  
1984); Morrison et al., Proc. Nat'l. Acad. Sci. USA, 81:6851-6855  
10 (1984); Morrison et al., Science, 229:1202-1207 (1985); Boulianne et  
al., Nature, 312:643-646 (13 December 1984); Capon et al., Nature,  
337:525-531 (1989); Traunecker et al., Nature, 339:68-70 (1989).

Alternatively, the chimeric molecules may be constructed as  
follows. The DNA including a region encoding the desired sequence, such  
15 as an Apo-2 and/or TNFR sequence, is cleaved by a restriction enzyme at  
or proximal to the 3' end of the DNA encoding the immunoglobulin-like  
domain(s) and at a point at or near the DNA encoding the N-terminal end  
of the Apo-2 or TNFR polypeptide (where use of a different leader is  
contemplated) or at or proximal to the N-terminal coding region for TNFR  
20 (where the native signal is employed). This DNA fragment then is  
readily inserted proximal to DNA encoding an immunoglobulin light or  
heavy chain constant region and, if necessary, the resulting construct  
tailored by deletional mutagenesis. Preferably, the Ig is a human  
immunoglobulin when the chimeric molecule is intended for *in vivo*  
25 therapy for humans. DNA encoding immunoglobulin light or heavy chain  
constant regions is known or readily available from cDNA libraries or is  
synthesized. See for example, Adams et al., Biochemistry, 19:2711-2719  
(1980); Gough et al., Biochemistry, 19:2702-2710 (1980); Dolby et al.,  
Proc. Natl. Acad. Sci. USA, 77:6027-6031 (1980); Rice et al., Proc.  
30 Natl. Acad. Sci., 79:7862-7865 (1982); Falkner et al., Nature, 298:286-  
288 (1982); and Morrison et al., Ann. Rev. Immunol., 2:239-256 (1984).

Further details of how to prepare such fusions are found in  
publications concerning the preparation of immunoadhesins.  
Immunoadhesins in general, and CD4-Ig fusion molecules specifically are  
35 disclosed in WO 89/02922, published 6 April 1989). Molecules comprising  
the extracellular portion of CD4, the receptor for human  
immunodeficiency virus (HIV), linked to IgG heavy chain constant region  
are known in the art and have been found to have a markedly longer half-  
life and lower clearance than the soluble extracellular portion of CD4  
40 [Capon et al., supra; Byrn et al., Nature, 344:667 (1990)]. The  
construction of specific chimeric TNFR-IgG molecules is also described  
in Ashkenazi et al. Proc. Natl. Acad. Sci., 88:10535-10539 (1991);

5 Lesslauer et al. [J. Cell. Biochem. Supplement 15F, 1991, p. 115 (P 432)]; and Peppel and Beutler, J. Cell. Biochem. Supplement 15F, 1991, p. 118 (P 439)].

B. Therapeutic and Non-therapeutic Uses for Apo-2

10 Apo-2, as disclosed in the present specification, can be employed therapeutically to induce apoptosis in mammalian cells. This therapy can be accomplished for instance, using *in vivo* or *ex vivo* gene therapy techniques and includes the use of the death domain sequences disclosed herein. The Apo-2 chimeric molecules (including the chimeric  
15 molecules containing an extracellular domain sequence of Apo-2) comprising immunoglobulin sequences can also be employed therapeutically to inhibit apoptosis or NF-KB induction by Apo-2L or by another ligand that Apo-2 binds to.

20 The Apo-2 of the invention also has utility in non-therapeutic applications. Nucleic acid sequences encoding the Apo-2 may be used as a diagnostic for tissue-specific typing. For example, procedures like *in situ* hybridization, Northern and Southern blotting, and PCR analysis may be used to determine whether DNA and/or RNA encoding Apo-2 is present in the cell type(s) being evaluated. Apo-2  
25 nucleic acid will also be useful for the preparation of Apo-2 by the recombinant techniques described herein.

30 The isolated Apo-2 may be used in quantitative diagnostic assays as a control against which samples containing unknown quantities of Apo-2 may be prepared. Apo-2 preparations are also useful in generating antibodies, as standards in assays for Apo-2 (e.g., by labeling Apo-2 for use as a standard in a radioimmunoassay, radioreceptor assay, or enzyme-linked immunoassay), in affinity purification techniques, and in competitive-type receptor binding assays when labeled with, for instance, radioiodine, enzymes, or fluorophores.

35 Modified forms of the Apo-2, such as the Apo-2-IgG chimeric molecules (immunoadhesins) described above, can be used as immunogens in producing anti-Apo-2 antibodies.

40 Nucleic acids which encode Apo-2 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or

5 rat) is an animal having cells that contain a transgene, which transgene  
was introduced into the animal or an ancestor of the animal at a  
prenatal, e.g., an embryonic stage. A transgene is a DNA which is  
integrated into the genome of a cell from which a transgenic animal  
develops. In one embodiment, cDNA encoding Apo-2 or an appropriate  
10 sequence thereof (such as Apo-2-IgG) can be used to clone genomic DNA  
encoding Apo-2 in accordance with established techniques and the genomic  
sequences used to generate transgenic animals that contain cells which  
express DNA encoding Apo-2. Methods for generating transgenic animals,  
particularly animals such as mice or rats, have become conventional in  
15 the art and are described, for example, in U.S. Patent Nos. 4,736,866  
and 4,870,009. Typically, particular cells would be targeted for Apo-2  
transgene incorporation with tissue-specific enhancers. Transgenic  
animals that include a copy of a transgene encoding Apo-2 introduced  
into the germ line of the animal at an embryonic stage can be used to  
20 examine the effect of increased expression of DNA encoding Apo-2. Such  
animals can be used as tester animals for reagents thought to confer  
protection from, for example, pathological conditions associated with  
excessive apoptosis. In accordance with this facet of the invention, an  
animal is treated with the reagent and a reduced incidence of the  
25 pathological condition, compared to untreated animals bearing the  
transgene, would indicate a potential therapeutic intervention for the  
pathological condition. In another embodiment, transgenic animals that  
carry a soluble form of Apo-2 such as an Apo-2 ECD or an immunoglobulin  
chimera of such form could be constructed to test the effect of chronic  
30 neutralization of Apo-2L, a ligand of Apo-2.

Alternatively, non-human homologues of Apo-2 can be used to  
construct an Apo-2 "knock out" animal which has a defective or altered  
gene encoding Apo-2 as a result of homologous recombination between the  
endogenous gene encoding Apo-2 and altered genomic DNA encoding Apo-2  
35 introduced into an embryonic cell of the animal. For example, cDNA  
encoding Apo-2 can be used to clone genomic DNA encoding Apo-2 in  
accordance with established techniques. A portion of the genomic DNA  
encoding Apo-2 can be deleted or replaced with another gene, such as a  
gene encoding a selectable marker which can be used to monitor  
40 integration. Typically, several kilobases of unaltered flanking DNA  
(both at the 5' and 3' ends) are included in the vector [see e.g.,  
Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous

5 recombination vectors]. The vector is introduced into an embryonic stem  
cell line (e.g., by electroporation) and cells in which the introduced  
DNA has homologously recombined with the endogenous DNA are selected  
[see e.g., Li et al., *Cell*, 69:915 (1992)]. The selected cells are then  
10 injected into a blastocyst of an animal (e.g., a mouse or rat) to form  
aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and  
Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL,  
Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted  
into a suitable pseudopregnant female foster animal and the embryo  
brought to term to create a "knock out" animal. Progeny harboring the  
15 homologously recombined DNA in their germ cells can be identified by  
standard techniques and used to breed animals in which all cells of the  
animal contain the homologously recombined DNA. Knockout animals can be  
characterized for instance, for their ability to defend against certain  
pathological conditions and for their development of pathological  
20 conditions due to absence of the Apo-2 polypeptide, including for  
example, development of tumors.

#### C. Anti-Apo-2 Antibody Preparation

The present invention further provides anti-Apo-2 antibodies.

25 Antibodies against Apo-2 may be prepared as follows. Exemplary  
antibodies include polyclonal, monoclonal, humanized, bispecific, and  
heteroconjugate antibodies.

##### 1. Polyclonal Antibodies

The Apo-2 antibodies may comprise polyclonal antibodies.

30 Methods of preparing polyclonal antibodies are known to the skilled  
artisan. Polyclonal antibodies can be raised in a mammal, for example,  
by one or more injections of an immunizing agent and, if desired, an  
adjuvant. Typically, the immunizing agent and/or adjuvant will be  
injected in the mammal by multiple subcutaneous or intraperitoneal  
35 injections. The immunizing agent may include the Apo-2 polypeptide or a  
fusion protein thereof. An example of a suitable immunizing agent is an  
Apo-2-IgG fusion protein, such as an Apo-2 ECD-IgG fusion protein.  
Cells expressing Apo-2 at their surface may also be employed. It may be  
useful to conjugate the immunizing agent to a protein known to be  
40 immunogenic in the mammal being immunized. Examples of such immunogenic  
proteins which may be employed include but are not limited to keyhole  
limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean



5 trypsin inhibitor. An aggregating agent such as alum may also be  
employed to enhance the mammal's immune response. Examples of adjuvants  
which may be employed include Freund's complete adjuvant and MPL-TDM  
adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).  
10 The immunization protocol may be selected by one skilled in the art  
without undue experimentation. The mammal can then be bled, and the  
serum assayed for antibody titer. If desired, the mammal can be boosted  
until the antibody titer increases or plateaus.

## 2. Monoclonal Antibodies

15 The Apo-2 antibodies may, alternatively, be monoclonal  
antibodies. Monoclonal antibodies may be prepared using hybridoma  
methods, such as those described by Kohler and Milstein, supra. In a  
hybridoma method, a mouse, hamster, or other appropriate host animal, is  
typically immunized (such as described above) with an immunizing agent  
to elicit lymphocytes that produce or are capable of producing  
20 antibodies that will specifically bind to the immunizing agent.  
Alternatively, the lymphocytes may be immunized *in vitro*.

25 The immunizing agent will typically include the Apo-2  
polypeptide or a fusion protein thereof. An example of a suitable  
immunizing agent is an Apo-2-IgG fusion protein or chimeric molecule. A  
specific example of an Apo-2 ECD-IgG immunogen is described in Example 9  
below. Cells expressing Apo-2 at their surface may also be employed.  
Generally, either peripheral blood lymphocytes ("PBLs") are used if  
cells of human origin are desired, or spleen cells or lymph node cells  
are used if non-human mammalian sources are desired. The lymphocytes  
30 are then fused with an immortalized cell line using a suitable fusing  
agent, such as polyethylene glycol, to form a hybridoma cell [Goding,  
Monoclonal Antibodies: Principles and Practice, Academic Press, (1986)  
pp. 59-103]. Immortalized cell lines are usually transformed mammalian  
cells, particularly myeloma cells of rodent, bovine and human origin.  
35 Usually, rat or mouse myeloma cell lines are employed. The hybridoma  
cells may be cultured in a suitable culture medium that preferably  
contains one or more substances that inhibit the growth or survival of  
the unfused, immortalized cells. For example, if the parental  
transformed cells lack the enzyme hypoxanthine guanine phosphoribosyl  
40 transferase (HGPRT or HPRT), the culture medium for the hybridomas  
typically will include hypoxanthine, aminopterin, and thymidine ("HAT  
medium"), which substances prevent the growth of HGPRT-deficient cells.

5 Preferred immortalized cell lines are those that fuse  
efficiently, support stable high level expression of antibody by the  
selected antibody-producing cells, and are sensitive to a medium such as  
HAT medium. More preferred immortalized cell lines are murine myeloma  
10 lines, which can be obtained, for instance, from the Salk Institute Cell  
Distribution Center, San Diego, California and the American Type Culture  
Collection, Rockville, Maryland. Human myeloma and mouse-human  
heteromyeloma cell lines also have been described for the production of  
human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984);  
15 Brodeur et al., Monoclonal Antibody Production Techniques and  
Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured  
can then be assayed for the presence of monoclonal antibodies directed  
against Apo-2. Preferably, the binding specificity of monoclonal  
antibodies produced by the hybridoma cells is determined by  
20 immunoprecipitation or by an *in vitro* binding assay, such as  
radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).  
Such techniques and assays are known in the art. The binding affinity  
of the monoclonal antibody can, for example, be determined by the  
Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220  
25 (1980).

After the desired hybridoma cells are identified, the clones  
may be subcloned by limiting dilution procedures and grown by standard  
methods [Goding, supra]. Suitable culture media for this purpose  
include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640  
30 medium. Alternatively, the hybridoma cells may be grown *in vivo* as  
ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be  
isolated or purified from the culture medium or ascites fluid by  
conventional immunoglobulin purification procedures such as, for  
35 example, protein A-Sepharose, hydroxylapatite chromatography, gel  
electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA  
methods, such as those described in U.S. Patent No. 4,816,567. DNA  
encoding the monoclonal antibodies of the invention can be readily  
40 isolated and sequenced using conventional procedures (e.g., by using  
oligonucleotide probes that are capable of binding specifically to genes  
encoding the heavy and light chains of murine antibodies). The

5 hybridoma cells of the invention serve as a preferred source of such  
DNA. Once isolated, the DNA may be placed into expression vectors,  
which are then transfected into host cells such as simian COS cells,  
Chinese hamster ovary (CHO) cells, or myeloma cells that do not  
10 otherwise produce immunoglobulin protein, to obtain the synthesis of  
monoclonal antibodies in the recombinant host cells. The DNA also may  
be modified, for example, by substituting the coding sequence for human  
heavy and light chain constant domains in place of the homologous murine  
sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by  
15 covalently joining to the immunoglobulin coding sequence all or part of  
the coding sequence for a non-immunoglobulin polypeptide. Such a non-  
immunoglobulin polypeptide can be substituted for the constant domains  
of an antibody of the invention, or can be substituted for the variable  
domains of one antigen-combining site of an antibody of the invention to  
create a chimeric bivalent antibody.

20 As described in the Examples below, anti-Apo-2 monoclonal  
antibodies have been prepared. One of these antibodies, 3F11.39.7, has  
been deposited with ATCC and has been assigned deposit accession no. HB-  
12456. In one embodiment, the monoclonal antibodies of the invention  
will have the same biological characteristics as the monoclonal  
25 antibodies secreted by the hybridoma cell line(s) deposited under  
Accession No. HB-12456. The term "biological characteristics" is used  
to refer to the *in vitro* and/or *in vivo* activities or properties of the  
monoclonal antibody, such as the ability to specifically bind to Apo-2  
or to substantially block, induce or enhance Apo-2 activation. As  
30 disclosed in the present specification, the 3F11.39.7 monoclonal  
antibody (HB-12456) is characterized as having agonistic activity for  
inducing apoptosis, binding to the Apo-2 receptor, having blocking  
activity as described in the Examples below, and having some cross-  
reactivity to DR4 but not to DcR1 or DcR2. Optionally, the monoclonal  
35 antibody will bind to the same epitope as the 3F11.39.7 antibody  
disclosed herein. This can be determined by conducting various assays,  
such as described herein and in the Examples. For instance, to  
determine whether a monoclonal antibody has the same specificity as the  
3F11.39.7 antibody specifically disclosed, one can compare activity in  
40 Apo-2 blocking and apoptosis induction assays, such as those described  
in the Examples below.

The antibodies of the invention may also comprise monovalent

5 antibodies. Methods for preparing monovalent antibodies are well known  
in the art. For example, one method involves recombinant expression of  
immunoglobulin light chain and modified heavy chain. The heavy chain is  
truncated generally at any point in the Fc region so as to prevent heavy  
chain crosslinking. Alternatively, the relevant cysteine residues are  
10 substituted with another amino acid residue or are deleted so as to  
prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent  
antibodies. Digestion of antibodies to produce fragments thereof,  
particularly, Fab fragments, can be accomplished using routine  
15 techniques known in the art. For instance, digestion can be performed  
using papain. Examples of papain digestion are described in WO 94/29348  
published 12/22/94 and U.S. Patent No. 4,342,566. Papain digestion of  
antibodies typically produces two identical antigen binding fragments,  
called Fab fragments, each with a single antigen binding site, and a  
20 residual Fc fragment. Pepsin treatment yields an F(ab')<sub>2</sub> fragment that  
has two antigen combining sites and is still capable of cross-linking  
antigen.

The Fab fragments produced in the antibody digestion also  
contain the constant domains of the light chain and the first constant  
25 domain (CH<sub>1</sub>) of the heavy chain. Fab' fragments differ from Fab  
fragments by the addition of a few residues at the carboxy terminus of  
the heavy chain CH<sub>1</sub> domain including one or more cysteines from the  
antibody hinge region. Fab'-SH is the designation herein for Fab' in  
which the cysteine residue(s) of the constant domains bear a free thiol  
30 group. F(ab')<sub>2</sub> antibody fragments originally were produced as pairs of  
Fab' fragments which have hinge cysteines between them. Other chemical  
couplings of antibody fragments are also known.

### 3. Humanized Antibodies

The Apo-2 antibodies of the invention may further comprise  
35 humanized antibodies or human antibodies. Humanized forms of non-human  
(e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin  
chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other  
antigen-binding subsequences of antibodies) which contain minimal  
sequence derived from non-human immunoglobulin. Humanized antibodies  
40 include human immunoglobulins (recipient antibody) in which residues  
from a complementary determining region (CDR) of the recipient are  
replaced by residues from a CDR of a non-human species (donor antibody)

5 such as mouse, rat or rabbit having the desired specificity, affinity  
and capacity. In some instances, Fv framework residues of the human  
immunoglobulin are replaced by corresponding non-human residues.  
Humanized antibodies may also comprise residues which are found neither  
10 in the recipient antibody nor in the imported CDR or framework  
sequences. In general, the humanized antibody will comprise  
substantially all of at least one, and typically two, variable domains,  
in which all or substantially all of the CDR regions correspond to those  
of a non-human immunoglobulin and all or substantially all of the FR  
regions are those of a human immunoglobulin consensus sequence. The  
15 humanized antibody optimally also will comprise at least a portion of an  
immunoglobulin constant region (Fc), typically that of a human  
immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et  
al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol.,  
2:593-596 (1992)].

20 Methods for humanizing non-human antibodies are well known in  
the art. Generally, a humanized antibody has one or more amino acid  
residues introduced into it from a source which is non-human. These  
non-human amino acid residues are often referred to as "import"  
residues, which are typically taken from an "import" variable domain.  
25 Humanization can be essentially performed following the method of Winter  
and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et  
al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-  
1536 (1988)], by substituting rodent CDRs or CDR sequences for the  
corresponding sequences of a human antibody. Accordingly, such  
30 "humanized" antibodies are chimeric antibodies (U.S. Patent No.  
4,816,567), wherein substantially less than an intact human variable  
domain has been substituted by the corresponding sequence from a non-  
human species. In practice, humanized antibodies are typically human  
antibodies in which some CDR residues and possibly some FR residues are  
35 substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy,  
to be used in making the humanized antibodies is very important in order  
to reduce antigenicity. According to the "best-fit" method, the  
sequence of the variable domain of a rodent antibody is screened against  
40 the entire library of known human variable domain sequences. The human  
sequence which is closest to that of the rodent is then accepted as the  
human framework (FR) for the humanized antibody [Sims et al., J.

5 Immunol., 151:2296 (1993); Chothia and Lesk, J. Mol. Biol., 196:901  
(1987)]. Another method uses a particular framework derived from the  
consensus sequence of all human antibodies of a particular subgroup of  
light or heavy chains. The same framework may be used for several  
different humanized antibodies [Carter et al., Proc. Natl. Acad. Sci.  
10 USA, 89:4285 (1992); Presta et al., J. Immunol., 151:2623 (1993)].

It is further important that antibodies be humanized with  
retention of high affinity for the antigen and other favorable  
biological properties. To achieve this goal, according to a preferred  
method, humanized antibodies are prepared by a process of analysis of  
15 the parental sequences and various conceptual humanized products using  
three dimensional models of the parental and humanized sequences. Three  
dimensional immunoglobulin models are commonly available and are  
familiar to those skilled in the art. Computer programs are available  
which illustrate and display probable three-dimensional conformational  
20 structures of selected candidate immunoglobulin sequences. Inspection  
of these displays permits analysis of the likely role of the residues in  
the functioning of the candidate immunoglobulin sequence, i.e., the  
analysis of residues that influence the ability of the candidate  
immunoglobulin to bind its antigen. In this way, FR residues can be  
25 selected and combined from the consensus and import sequence so that the  
desired antibody characteristic, such as increased affinity for the  
target antigen(s), is achieved. In general, the CDR residues are  
directly and most substantially involved in influencing antigen binding  
[see, WO 94/04679 published 3 March 1994].

30 Transgenic animals (e.g., mice) that are capable, upon  
immunization, of producing a full repertoire of human antibodies in the  
absence of endogenous immunoglobulin production can be employed.  
Transfer of the human germ-line immunoglobulin gene array in such germ-  
line mutant mice will result in the production of human antibodies upon  
35 antigen challenge [see, e.g., Jakobovits et al., Proc. Natl. Acad. Sci.  
USA, 90:2551-255 (1993); Jakobovits et al., Nature, 362:255-258 (1993);  
Bruggemann et al., Year in Immuno., 7:33 (1993)]. Human antibodies can  
also be produced in phage display libraries [Hoogenboom and Winter, J.  
Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581  
40 (1991)]. The techniques of Cole et al. and Boerner et al. are also  
available for the preparation of human monoclonal antibodies (Cole et  
al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77

5 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

#### 4. Bispecific Antibodies

10 Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the Apo-2, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

15 Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

25 According to a different and more preferred approach, antibody variable domains with the desired binding specificities (antibody-antigen combining sites) are fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. This provides for great flexibility in adjusting the mutual proportions of the three polypeptide fragments in embodiments when unequal ratios of the three polypeptide chains used in the construction provide the optimum yields. It is, however, possible to insert the coding sequences for two or all three polypeptide chains in one expression vector when the expression of at least two polypeptide chains in equal ratios results in high yields or when the ratios are of no particular significance. In a preferred

embodiment of this approach, the bispecific antibodies are composed of a hybrid immunoglobulin heavy chain with a first binding specificity in one arm, and a hybrid immunoglobulin heavy-chain/light-chain pair (providing a second binding specificity) in the other arm. It was found that this asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation. This approach is disclosed in WO 94/04690 published 3 March 1994. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [US Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Pat. No. 4,676,980.

#### 6. Triabodies

Triabodies are also within the scope of the invention. Such antibodies are described for instance in Iliades et al., FEBS Letters, 409:437-441 (1997) and Korrt et al., Protein Engineering, 10:423-433 (1997).

#### 7. Other Modifications

Other modifications of the Apo-2 antibodies are contemplated. For example, it may be desirable to modify the antibodies of the invention with respect to effector function, so as to enhance the therapeutic effectiveness of the antibodies. For instance, cysteine residue(s) may be introduced into the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing [see, e.g., Caron et



5 al., J. Exp. Med., 176:1191-1195 (1992); Shopes, J. Immunol., 148:2918-  
2922 (1992). Homodimeric antibodies may also be prepared using  
heterobifunctional cross-linkers as described in Wolff et al., Cancer  
Research, 53:2560-2565 (1993). Ghetie et al., Proc. Natl. Acad. Sci.,  
10 94:7509-7514 (1997), further describe preparation of IgG-IgG homodimers  
and disclose that such homodimers can enhance apoptotic activity as  
compared to the monomers. Alternatively, the antibodies can be  
engineered to have dual Fc regions [see, Stevenson et al., Anti-Cancer  
Drug Design, 3:219-230 (1989)].

15 D. Therapeutic and Non-therapeutic Uses for Apo-2 Antibodies

The Apo-2 antibodies of the invention have therapeutic  
utility. Agonistic Apo-2 antibodies, for instance, may be employed to  
activate or stimulate apoptosis in cancer cells. The Apo-2 antibodies  
of the invention may also be useful in enhancing immune-mediated cell  
20 death in cells expressing Apo-2, for instance, through complement  
fixation or ADCC. Alternatively, antagonistic antibodies may be used to  
block excessive apoptosis (for instance in neurodegenerative disease) or  
to block potential autoimmune/inflammatory effects of Apo-2 resulting  
from NF-KB activation.

25 Apo-2 antibodies may further be used in diagnostic assays for  
Apo-2, e.g., detecting its expression in specific cells, tissues, or  
serum. Various diagnostic assay techniques known in the art may be  
used, such as competitive binding assays, direct or indirect sandwich  
assays and immunoprecipitation assays conducted in either heterogeneous  
30 or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of  
Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in  
the diagnostic assays can be labeled with a detectable moiety. The  
detectable moiety should be capable of producing, either directly or  
indirectly, a detectable signal. For example, the detectable moiety may  
35 be a radioisotope, such as <sup>3</sup>H, <sup>14</sup>C, <sup>32</sup>P, <sup>35</sup>S, or <sup>125</sup>I, a fluorescent or  
chemiluminescent compound, such as fluorescein isothiocyanate,  
rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase,  
beta-galactosidase or horseradish peroxidase. Any method known in the  
art for conjugating the antibody to the detectable moiety may be  
40 employed, including those methods described by Hunter et al., Nature,  
144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et

5 al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

10 Apo-2 antibodies also are useful for the affinity purification of Apo-2 from recombinant cell culture or natural sources. In this process, the antibodies against Apo-2 are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the Apo-2 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the Apo-2, which is bound to the  
15 immobilized antibody. Finally, the support is washed with another suitable solvent that will release the Apo-2 from the antibody.

#### E. Kits Containing Apo-2 or Apo-2 Antibodies

20 In a further embodiment of the invention, there are provided articles of manufacture and kits containing Apo-2 or Apo-2 antibodies which can be used, for instance, for the therapeutic or non-therapeutic applications described above. The article of manufacture comprises a container with a label. Suitable containers include, for example, bottles, vials, and test tubes. The containers may be formed from  
25 a variety of materials such as glass or plastic. The container holds a composition which includes an active agent that is effective for therapeutic or non-therapeutic applications, such as described above. The active agent in the composition is Apo-2 or an Apo-2 antibody. The label on the container indicates that the composition is used for a  
30 specific therapy or non-therapeutic application, and may also indicate directions for either *in vivo* or *in vitro* use, such as those described above.

35 The kit of the invention will typically comprise the container described above and one or more other containers comprising materials desirable from a commercial and user standpoint, including buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

\*\*\*\*\*

40 The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present

5 specification are hereby incorporated by reference in their entirety.

#### EXAMPLES

10 All restriction enzymes referred to in the examples were purchased from New England Biolabs and used according to manufacturer's instructions. All other commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

15

#### EXAMPLE 1

##### Isolation of cDNA clones Encoding Human Apo-2

20 Expressed sequence tag (EST) DNA databases (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) were searched and an EST was identified which showed homology to the death domain of the Apo-3 receptor [Marsters et al., Curr. Biol., 6:750 (1996)]. Human pancreas and kidney lgt10 bacteriophage cDNA libraries (both purchased from Clontech) were ligated into pRK5 vectors as follows. Reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (3 ml); pRK5, Xho1, 25 Not1 digested vector, 0.5 mg, 1 ml); cDNA (5 ml) and distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) were added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1). The aqueous phase was removed, collected and diluted into 5M NaCl (10 ml) and absolute ethanol 30 (-20°C, 250 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, and the pellet resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The DNA pellet was then dried in a speedvac and eluted into distilled water (3 ml) for use in the subsequent procedure.

35 The ligated cDNA/pRK5 vector DNA prepared previously was chilled on ice to which was added electrocompetent DH10B bacteria (Life Tech., 20 ml). The bacteria vector mixture was then electroporated as per the manufacturers recommendation. Subsequently SOC media (1 ml) was added and the mixture was incubated at 37°C for 30 minutes. The 40 transformants were then plated onto 20 standard 150 mm LB plates containing ampicillin and incubated for 16 hours (37°C) to allow the

5 colonies to grow. Positive colonies were then scraped off and the DNA isolated from the bacterial pellet using standard CsCl-gradient protocols.

10 An enriched 5'-cDNA library was then constructed to obtain a bias of cDNA fragments which preferentially represents the 5' ends of cDNA's contained within the library. 10 mg of the pooled isolated full-length library plasmid DNA (41 ml) was combined with Not 1 restriction buffer (New England Biolabs, 5 ml) and Not 1 (New England Biolabs, 4 ml) and incubated at 37°C for one hour. The reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml), the aqueous phase removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml). This was then centrifuged for 20 minutes at 14,000 x g, decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was then removed, the pellet dried in a speedvac and resuspended in distilled water (10 ml).

20 The following reagents were brought together and incubated at 37°C for 2 hours: distilled water (3 ml); linearized DNA library (1 mg, 1 ml); Ribonucleotide mix (Invitrogen, 10 ml); transcription buffer (Invitrogen, 2 ml) and Sp6 enzyme mix. The reaction was then extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 50 ml) and the aqueous phase was removed, collected and resuspended into 5M NaCl (5 ml) and absolute ethanol (-20°C, 150 ml) and centrifuged for 20 minutes at 14,000 x g. The pellet was then decanted and resuspended in 70% ethanol (0.5 ml), centrifuged again for 2 minutes at 14,000 x g, decanted, dried in a speedvac and resuspended into distilled water (10 ml).

30 The following reagents were added together and incubated at 16°C for 16 hours: 5X T4 ligase buffer (Life Tech., 3 ml); pRK5 Cla-Sal digested vector, 0.5 mg, 1 ml); cDNA (5 ml); distilled water (6 ml). Subsequently, additional distilled water (70 ml) and 10 mg/ml tRNA (0.1 ml) was added and the entire reaction was extracted through phenol:chloroform:isoamyl alcohol (25:24:1, 100 ml). The aqueous phase was removed, collected and diluted by 5M NaCl (10 ml) and absolute ethanol (-20°C, 250 ml) and centrifuged for 20 minutes at 14,000 x g. The DNA pellet was decanted, resuspended into 70% ethanol (0.5 ml) and centrifuged again for 2 minutes at 14,000 x g. The supernatant was

5 removed and the residue pellet was dried in a speedvac and resuspended  
in distilled water (3 ml). The ligated cDNA/pSST-amy.1 vector DNA was  
chilled on ice to which was added electrocompetent DH10B bacteria (Life  
Tech., 20 ml). The bacteria vector mixture was then electroporated as  
10 recommended by the manufacturer. Subsequently, SOC media (Life Tech., 1  
ml) was added and the mixture was incubated at 37°C for 30 minutes. The  
transformants were then plated onto 20 standard 150 mm LB plates  
containing ampicillin and incubated for 16 hours (37°C). Positive  
colonies were scraped off the plates and the DNA was isolated from the  
bacterial pellet using standard protocols, e.g. CsCl-gradient.

15 The cDNA libraries were screened by hybridization with a  
synthetic oligonucleotide probe:  
GGGAGCCGCTCATGAGGAAGTTGGGCCTCATGGACAATGAGATAAAGGTGGCTAAAGCTGAGGCAGCGGG  
(SEQ ID NO:3) based on the EST.

20 Three cDNA clones were sequenced in entirety. The  
overlapping coding regions of the cDNAs were identical except for codon  
410 (using the numbering system for Fig. 1); this position encoded a  
leucine residue (TTG) in both pancreatic cDNAs, and a methionine residue  
(ATG) in the kidney cDNA, possibly due to polymorphism.

25 The entire nucleotide sequence of Apo-2 is shown in Figure 1  
(SEQ ID NO:2). Clone 27868 (also referred to as pRK5-Apo-2 deposited as  
ATCC 209021, as indicated below) contains a single open reading frame  
with an apparent translational initiation site at nucleotide positions  
140-142 [Kozak et al., supra] and ending at the stop codon found at  
nucleotide positions 1373-1375 (Fig. 1; SEQ ID NO:2). The predicted  
30 polypeptide precursor is 411 amino acids long, a type I transmembrane  
protein, and has a calculated molecular weight of approximately 45 kDa.  
Hydropathy analysis (not shown) suggested the presence of a signal  
sequence (residues 1-53), followed by an extracellular domain (residues  
54-182), a transmembrane domain (residues 183-208), and an intracellular  
35 domain (residues 209-411) (Fig. 2A; SEQ ID NO:1). N-terminal amino acid  
sequence analysis of Apo-2-IgG expressed in 293 cells showed that the  
mature polypeptide starts at amino acid residue 54, indicating that the  
actual signal sequence comprises residues 1-53. Apo-2 polypeptide is  
obtained or obtainable by expressing the molecule encoded by the cDNA  
40 insert of the deposited ATCC 209021 vector.

TNF receptor family proteins are typically characterized by

5 the presence of multiple (usually four) cysteine-rich domains in their extracellular regions -- each cysteine-rich domain being approximately 45 amino acids long and containing approximately 6, regularly spaced, cysteine residues. Based on the crystal structure of the type 1 TNF receptor, the cysteines in each domain typically form three disulfide  
10 bonds in which usually cysteines 1 and 2, 3 and 5, and 4 and 6 are paired together. Like DR4, Apo-2 contains two extracellular cysteine-rich pseudorepeats (Fig. 2A), whereas other identified mammalian TNFR family members contain three or more such domains [Smith et al., Cell, 76:959 (1994)].

15 The cytoplasmic region of Apo-2 contains a death domain (amino acid residues 324-391 shown in Fig. 1; see also Fig. 2A) which shows significantly more amino acid sequence identity to the death domain of DR4 (64%) than to the death domain of TNFR1 (30%); CD95 (19%); or Apo-3/DR3 (29%) (Fig. 2B). Four out of six death domain amino acids that are required for signaling by TNFR1 [Tartaglia et al., supra] are conserved in Apo-2 while the other two residues are semi-conserved (see Fig. 2B).

20 Based on an alignment analysis (using the ALIGN<sup>TM</sup> computer program) of the full-length sequence, Apo-2 shows more sequence identity to DR4 (55%) than to other apoptosis-linked receptors, such as TNFR1 (19%); CD95 (17%); or Apo-3 (also referred to as DR3, WSL-1 or TRAMP) (29%).

## EXAMPLE 2

### A. Expression of Apo-2 ECD

30 A soluble extracellular domain (ECD) fusion construct was prepared. An Apo-2 ECD (amino acid residues 1-184 shown in Figure 1) was obtained by PCR and fused to a C-terminal Flag epitope tag (Sigma). (The Apo-2 ECD construct included residues 183 and 184 shown in Figure 1  
35 to provide flexibility at the junction, even though residues 183 and 184 are predicted to be in the transmembrane region). The Flag epitope-tagged molecule was then inserted into pRK5, and expressed by transient transfection into human 293 cells (ATCC CRL 1573).

40 After a 48 hour incubation, the cell supernatants were collected and either used directly for co-precipitation studies (see Example 3) or subjected to purification of the Apo-2 ECD-Flag by affinity chromatography on anti-Flag agarose beads, according to

5 manufacturer's instructions (Sigma).

#### B. Expression of Apo-2 ECD as an Immunoadhesin

10 A soluble Apo-2 ECD immunoadhesin construct was prepared. The Apo-2 ECD (amino acids 1-184 shown in Fig. 1) was fused to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al.,  
15 supra.

#### EXAMPLE 3

##### Immunoprecipitation Assay Showing Binding Interaction Between Apo-2 and Apo-2 Ligand

20 To determine whether Apo-2 and Apo-2L interact or associate with each other, supernatants from mock-transfected 293 cells or from 293 cells transfected with Apo-2 ECD-Flag (described in Example 2 above) (5 ml) were incubated with 5 µg poly-histidine-tagged soluble Apo-2L [Pitti et al., supra] for 30 minutes at room temperature and then  
25 analyzed for complex formation by a co-precipitation assay.

The samples were subjected to immunoprecipitation using 25 µl anti-Flag conjugated agarose beads (Sigma) or Nickel-conjugated agarose beads (Qiagen). After a 1.5 hour incubation at 4° C, the beads were spun down and washed four times in phosphate buffered saline (PBS). By  
30 using anti-Flag agarose, the Apo-2L was precipitated through the Flag-tagged Apo-2 ECD; by using Nickel-agarose, the Apo-2 ECD was precipitated through the His-tagged Apo-2L. The precipitated proteins were released by boiling the beads for 5 minutes in SDS-PAGE buffer, resolved by electrophoresis on 12% polyacrylamide gels, and then  
35 detected by immunoblot with anti-Apo-2L or anti-Flag antibody (2 µg/ml) as described in Marsters et al., J. Biol. Chem., (1997).

The results, shown in Figure 3, indicate that the Apo-2 ECD and Apo-2L can associate with each other.

40 The binding interaction was further analyzed by purifying Apo-2 ECD from the transfected 293 cell supernatants with anti-Flag beads (see Example 2) and then analyzing the samples on a BIAcore™

5 instrument. The BIAcore™ analysis indicated a dissociation constant ( $K_d$ ) of about 1 nM. BIAcore™ analysis also showed that the Apo-2 ECD is not capable of binding other apoptosis-inducing TNF family members, namely, TNF-alpha (Genentech, Inc., Pennica et al., Nature, 312:712 (1984), lymphotoxin-alpha (Genentech, Inc.), or Fas/Apo-1 ligand (Alexis Biochemicals). The data thus shows that Apo-2 is a specific receptor for Apo-2L.

#### EXAMPLE 4

##### Induction of Apoptosis by Apo-2

15 Because death domains can function as oligomerization interfaces, over-expression of receptors that contain death domains may lead to activation of signaling in the absence of ligand [Frazer et al., supra, Nagata et al., supra]. To determine whether Apo-2 was capable of inducing cell death, human 293 cells or HeLa cells (ATCC CCL 2.2) were transiently transfected by calcium phosphate precipitation (293 cells) or electroporation (HeLa cells) with a pRK5 vector or pRK5-based plasmids encoding Apo-2 and/or CrmA. When applicable, the total amount of plasmid DNA was adjusted by adding vector DNA. Apoptosis was assessed 24 hours after transfection by morphology (Fig. 4A); DNA fragmentation (Fig. 4B); or by FACS analysis of phosphatidylserine exposure (Fig. 4C) as described in Marsters et al., Curr. Biol., 6:1669 (1996). As shown in Figs. 4A and 4B, the Apo-2 transfected 293 cells underwent marked apoptosis.

25 For samples assayed by FACS, the HeLa cells were co-transfected with pRK5-CD4 as a marker for transfection and apoptosis was determined in CD4-expressing cells; FADD was co-transfected with the Apo-2 plasmid; the data are means  $\pm$  SEM of at least three experiments, as described in Marsters et al., Curr. Biol., 6:1669 (1996). The caspase inhibitors, DEVD-fmk (Enzyme Systems) or z-VAD-fmk (Research Biochemicals Intl.) were added at 200  $\mu$ M at the time of transfection. As shown in Fig. 4C, the caspase inhibitors CrmA, DEVD-fmk, and z-VAD-fmk blocked apoptosis induction by Apo-2, indicating the involvement of Ced-3-like proteases in this response.

35 FADD is an adaptor protein that mediates apoptosis activation by CD95, TNFR1, and Apo-3/DR3 [Nagata et al., supra], but does not appear necessary for apoptosis induction by Apo-2L [Marsters et



5 al., supra] or by DR4 [Pan et al., supra]. A dominant-negative mutant  
form of FADD, which blocks apoptosis induction by CD95, TNFR1, or Apo-  
3/DR3 [Frazer et al., supra; Nagata et al., supra; Chinnayian et al.,  
supra] did not inhibit apoptosis induction by Apo-2 when co-transfected  
10 signals apoptosis independently of FADD. Consistent with this  
conclusion, a glutathione-S-transferase fusion protein containing the  
Apo-2 cytoplasmic region did not bind to *in vitro* transcribed and  
translated FADD (data not shown).

#### 15 EXAMPLE 5

##### Inhibition of Apo-2L Activity by Soluble Apo-2 ECD

Soluble Apo-2L (0.5 µg/ml, prepared as described in Pitti et  
al., supra) was pre-incubated for 1 hour at room temperature with PBS  
buffer or affinity-purified Apo-2 ECD (5 µg/ml) together with anti-Flag  
20 antibody (Sigma) (1 µg/ml) and added to HeLa cells. After a 5 hour  
incubation, the cells were analyzed for apoptosis by FACS (as above)  
(Fig. 4D).

Apo-2L induced marked apoptosis in HeLa cells, and the  
soluble Apo-2 ECD was capable of blocking Apo-2L action (Fig. 4D),  
25 confirming a specific interaction between Apo-2L and Apo-2. Similar  
results were obtained with the Apo-2 ECD immunoadhesin (Fig. 4D). Dose-  
response analysis showed half-maximal inhibition at approximately 0.3 nM  
Apo-2 immunoadhesin (Fig. 4E).

#### 30 EXAMPLE 6

##### Activation of NF-KB by Apo-2

An assay was conducted to determine whether Apo-2 activates  
NF-KB.

HeLa cells were transfected with pRK5 expression plasmids  
35 encoding full-length native sequence Apo-2, DR4 or Apo-3 and harvested  
24 hours after transfection. Nuclear extracts were prepared and 1 µg of  
nuclear protein was reacted with a <sup>32</sup>P-labelled NF-KB-specific synthetic  
oligonucleotide probe

ATCAGGGACTTTCCGCTGGGGACTTTCCG (SEQ ID NO:4) [see, also, MacKay et al.,  
40 J. Immunol., 153:5274-5284 (1994)], alone or together with a 50-fold

5 excess of unlabelled probe, or with an irrelevant <sup>32</sup>P-labelled synthetic oligonucleotide

AGGATGGGAAGTGTGTGATATATCCTTGAT (SEQ ID NO:5). In some samples, antibody to p65/RelA subunits of NF-KB (1 µg/ml; Santa Cruz Biotechnology) was added. DNA binding was analyzed by an  
10 electrophoretic mobility shift assay as described by Hsu et al., supra; Marsters et al., supra, and MacKay et al., supra.

The results are shown in Fig. 5. As shown in Fig. 5A, upon transfection into HeLa cells, both Apo-2 and DR4 induced significant NF-KB activation as measured by the electrophoretic mobility shift assay;  
15 the level of activation was comparable to activation observed for Apo-3/DR3. Antibody to the p65/RelA subunit of NF-KB inhibited the mobility of the NF-KB probe, implicating p65 in the response to all 3 receptors.

An assay was also conducted to determine if Apo-2L itself can regulate NF-KB activity. HeLa cells or MCF7 cells (human breast  
20 adenocarcinoma cell line, ATCC HTB 22) were treated with PBS buffer, soluble Apo-2L (Pitti et al., supra) or TNF-alpha (Genentech, Inc., see Pennica et al., Nature, 312:721 (1984)) (1 µg/ml) and assayed for NF-KB activity as above. The results are shown in Fig. 5B. The Apo-2L induced a significant NF-KB activation in the treated HeLa cells but not  
25 in the treated MCF7 cells; the TNF-alpha induced a more pronounced activation in both cell lines. Several studies have disclosed that NF-KB activation by TNF can protect cells against TNF-induced apoptosis [Nagata, supra].

The effects of a NF-KB inhibitor, ALLN (N-acetyl-Leu-Leu-norleucinal) and a transcription inhibitor, cyclohexamide, were also  
30 tested. The HeLa cells (plated in 6-well dishes) were preincubated with PBS buffer, ALLN (Calbiochem) (40 µg/ml) or cyclohexamide (Sigma) (50 µg/ml) for 1 hour before addition of Apo-2L (1 µg/ml). After a 5 hour incubation, apoptosis was analyzed by FACS (see Fig. 5C).

35 The results are shown in Fig. 5C. Both ALLN and cyclohexamide increased the level of Apo-2L-induced apoptosis in the HeLa cells. The data indicates that Apo-2L can induce protective NF-KB-dependent genes. The data also indicates that Apo-2L is capable of

5 activating NF-KB in certain cell lines and that both Apo-2 and DR4 may mediate that function.

#### EXAMPLE 7

##### Northern Blot Analysis

10 Expression of Apo-2 mRNA in human tissues was examined by Northern blot analysis. Human RNA blots were hybridized to a 4.6 kilobase <sup>32</sup>P-labelled DNA probe based on the full length Apo-2 cDNA; the probe was generated by digesting the pRK5-Apo-2 plasmid with EcoRI. Human fetal RNA blot MTN (Clontech) and human adult RNA blot MTN-II  
15 (Clontech) were incubated with the DNA probes. Blots were incubated with the probes in hybridization buffer (5X SSPE; 2X Denhardt's solution; 100 mg/mL denatured sheared salmon sperm DNA; 50% formamide; 2% SDS) for 60 hours at 42°C. The blots were washed several times in 2X SSC; 0.05% SDS for 1 hour at room temperature, followed by a 30 minute  
20 wash in 0.1X SSC; 0.1% SDS at 50°C. The blots were developed after overnight exposure.

As shown in Fig. 6, a predominant mRNA transcript of approximately 4.6kb was detected in multiple tissues. Expression was relatively high in fetal and adult liver and lung, and in adult ovary  
25 and peripheral blood leukocytes (PBL), while no mRNA expression was detected in fetal and adult brain. Intermediate levels of expression were seen in adult colon, small intestine, testis, prostate, thymus, pancreas, kidney, skeletal muscle, placenta, and heart. Several adult tissues that express Apo-2, e.g., PBL, ovary, and spleen, have been  
30 shown previously to express DR4 [Pan et al., supra], however, the relative levels of expression of each receptor mRNA appear to be different.

#### EXAMPLE 8

##### Chromosomal Localization of the Apo-2 gene

35 Chromosomal localization of the human Apo-2 gene was examined by radiation hybrid (RH) panel analysis. RH mapping was performed by PCR using a human-mouse cell radiation hybrid panel (Research Genetics) and primers based on the coding region of the Apo-2 cDNA [Gelb et al.,  
40 Hum. Genet., 98:141 (1996)]. Analysis of the PCR data using the Stanford Human Genome Center Database indicates that Apo-2 is linked to

5 the marker D8S481, with an LOD of 11.05; D8S481 is linked in turn to D8S2055, which maps to human chromosome 8p21. A similar analysis of DR4 showed that DR4 is linked to the marker D8S2127 (with an LOD of 13.00), which maps also to human chromosome 8p21.

10 To Applicants' present knowledge, to date, no other member of the TNFR gene family has been located to chromosome 8.

#### EXAMPLE 9

##### Preparation of Monoclonal Antibodies Specific for Apo-2

15 Balb/c mice (obtained from Charles River Laboratories) were immunized by injecting 0.5µg/50µl of an Apo-2 ECD immunoadhesin protein (diluted in MPL-TDM adjuvant purchased from Ribi Immunochemical Research Inc., Hamilton, MT) 11 times into each hind foot pad at 3-4 day intervals. The Apo-2 ECD immunoadhesin protein was generated by fusing an extracellular domain sequence of Apo-2 (amino acids 1-184 shown in Fig. 1) to the hinge and Fc region of human immunoglobulin G<sub>1</sub> heavy chain in pRK5 as described previously [Ashkenazi et al., Proc. Natl. Acad. Sci., 88:10535-10539 (1991)]. The immunoadhesin protein was expressed by transient transfection into human 293 cells and purified from cell supernatants by protein A affinity chromatography, as described by Ashkenazi et al., supra (See also Example 2B above).

25 Three days after the final boost, popliteal lymph nodes were removed from the mice and a single cell suspension was prepared in DMEM media (obtained from Biowhitakker Corp.) supplemented with 1% penicillin-streptomycin. The lymph node cells were then fused with murine myeloma cells P3X63AgU.1 (ATCC CRL 1597) using 35% polyethylene glycol and cultured in 96-well culture plates. Hybridomas resulting from the fusion were selected in HAT medium. Ten days after the fusion, hybridoma culture supernatants were screened in an ELISA to test for the presence of monoclonal antibodies binding to the Apo-2 ECD immunoadhesin protein.

35 In the ELISA, 96-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated by adding 50 µl of 2 µg/ml goat anti-human IgG Fc (purchased from Cappel Laboratories) in PBS to each well and incubating at 4°C overnight. The plates were then washed three times with wash buffer (PBS containing 0.05% Tween 20). The wells in the microtiter plates were then blocked with 50 µl of 2.0% bovine serum

5 albumin in PBS and incubated at room temperature for 1 hour. The plates were then washed again three times with wash buffer.

After the washing step, 50  $\mu$ l of 0.4  $\mu$ g/ml Apo-2 ECD immunoadhesin protein (as described above) in assay buffer was added to each well. The plates were incubated for 1 hour at room temperature on a shaker apparatus, followed by washing three times with wash buffer.

Following the wash steps, 100  $\mu$ l of the hybridoma supernatants or purified antibody (using Protein A-sepharose columns) (1  $\mu$ g/ml) was added to designated wells in the presence of CD4-IgG. 100  $\mu$ l of P3X63AgU.1 myeloma cell conditioned medium was added to other designated wells as controls. The plates were incubated at room temperature for 1 hour on a shaker apparatus and then washed three times with wash buffer.

Next, 50  $\mu$ l HRP-conjugated goat anti-mouse IgG Fc (purchased from Cappel Laboratories), diluted 1:1000 in assay buffer (0.5% bovine serum albumin, 0.05% Tween-20, 0.01% Thimersol in PBS), was added to each well and the plates incubated for 1 hour at room temperature on a shaker apparatus. The plates were washed three times with wash buffer, followed by addition of 50  $\mu$ l of substrate (TMB microwell peroxidase substrate, Kirkegaard & Perry, Gaithersburg, MD) to each well and incubation at room temperature for 10 minutes. The reaction was stopped by adding 50  $\mu$ l of TMB 1-component stop solution (diethyl glycol, Kirkegaard & Perry) to each well, and absorbance at 450 nm was read in an automated microtiter plate reader.

Of the hybridoma supernatants screened in the ELISA, 22 supernatants tested positive (calculated as approximately 4 times above background). The supernatants testing positive in the ELISA were further analyzed by FACS analysis using 9D cells (a human B lymphoid cell line expressing Apo-2; Genentech, Inc.) and FITC-conjugated goat anti-mouse IgG. For this analysis, 25  $\mu$ l of cells suspended (at  $4 \times 10^6$  cells/ml) in cell sorter buffer (PBS containing 1% FCS and 0.02%  $\text{NaN}_3$ ) were added to U-bottom microtiter wells, mixed with 100  $\mu$ l of culture supernatant or purified antibody (purified on Protein A-sepharose columns) (10  $\mu$ g /ml) in cell sorter buffer, and incubated for 30 minutes on ice. The cells were then washed and incubated with 100  $\mu$ l FITC-

5 conjugated goat anti-mouse IgG for 30 minutes at 4°C. Cells were then washed twice, resuspended in 150 µl of cell sorter buffer and then analyzed by FACScan (Becton Dickinson, Mountain View, CA). FACS analysis showed 8/22 supernatants were positive for anti-Apo-2 antibodies.

10 Figure 7 shows the FACS staining of 9D cells incubated with one of the Apo-2 antibodies, referred to as 3F11.39.7. As shown in Figure 7, the 3F11.39.7 antibody recognizes the Apo-2 receptor expressed in 9D cells.

#### 15 EXAMPLE 10

##### Assay for Ability of Apo-2 Abs to Agonistically induce Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to induce Apo-2 mediated 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were incubated with varying concentrations of antibodies in 100 µl complete RPMI media at 4°C for 15 minutes. The cells were then incubated for 5 minutes at 37°C and 10 µg of goat anti-mouse IgG Fc antibody (Cappel Laboratories) in 300 µl of complete RPMI was added to some of the cell samples. At this point, the cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). The cells were washed in PBS and resuspended in 200 µl binding buffer. Ten µl of annexin-V-FITC (1 µg/ml) and 10 µl of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

As shown in Figure 8, the 3F11.39.7 antibody (in the absence of the goat anti-mouse IgG Fc) induced apoptosis in the 9D cells as compared to the control antibodies. Agonistic activity, however, was enhanced by Apo-2 receptor cross-linking in the presence of the goat anti-mouse IgG Fc (see Figure 9). This enhanced apoptosis (Figure 9) by the combination of antibodies is comparable to the apoptotic activity of Apo-2L in 9D cells (data not shown).

Assay for Antibody Ability to Block Apo-2 ligand-induced Apoptosis

Hybridoma supernatants and purified antibodies (as described in Example 9 above) were tested for activity to block Apo-2 ligand induced 9D cell apoptosis. The 9D cells ( $5 \times 10^5$  cells/0.1ml) were suspended in complete RPMI media (RPMI plus 10%FCS, glutamine, nonessential amino acids, penicillin, streptomycin, sodium pyruvate) and placed into individual Falcon 2052 tubes. Cells were then incubated with 10  $\mu$ g of antibodies in 200  $\mu$ l media for 15 minutes on ice. 0.2 ml of Apo-2 ligand (2.5  $\mu$ g/ml) (soluble His-tagged Apo-2L prepared as described in WO 97/25428; see also Pitti et al., *supra*) was suspended into complete RPMI media, and then added into the tubes containing the 9D cells. The 9D cells were incubated overnight at 37°C and in the presence of 7% CO<sub>2</sub>. The incubated cells were then harvested and washed once with PBS. The viability of the cells was determined by staining of FITC-annexin V binding to phosphatidylserine according to manufacturer recommendations (Clontech). Specifically, the cells were washed in PBS and resuspended in 200  $\mu$ l binding buffer. Ten  $\mu$ l of annexin-V-FITC (1  $\mu$ g/ml) and 10  $\mu$ l of propidium iodide were added to the cells. After incubation for 15 minutes in the dark, the 9D cells were analyzed by FACS.

The results are shown in Figure 10. Since 9D cells express more than one receptor for Apo-2L, Apo-2L can induce apoptosis in the 9D cells by interacting with either Apo-2 or the DR4 receptor. Thus, to detect any blocking activity of the Apo-2 antibodies, the interaction between DR4 and Apo-2L needed to be blocked. In combination with the anti-DR4 antibody, 4H6.17.8 (ATCC HB-12455), the Apo-2 antibody 3F11.39.7 was able to block approximately 50% of apoptosis induced by Apo-2L. The remaining approximately 50% apoptotic activity is believed to be due to the agonistic activities of these two antibodies by themselves, as shown in Figure 10. Accordingly, it is believed that the 3F11.39.7 antibody is a blocking Apo-2 antibody.

5

#### EXAMPLE 12

##### ELISA Assay to Test Binding of Apo-2 Antibodies to Other

##### Apo-2 Ligand Receptors

10 An ELISA was conducted to determine if the monoclonal antibody described in Example 9 was able to bind other known Apo-2L receptors beside Apo-2. Specifically, the 3F11.39.7 antibody was tested for binding to DR4 [Pan et al., supra], DcR1 [Sheridan et al., supra], and DcR2 [Marsters et al., Curr. Biol., 7:1003-1006 (1997)]. The ELISA was performed essentially as described in Example 9 above.

15 The results are shown in Figure 11. The Apo-2 antibody 3F11.39.7 bound to Apo-2. The 3F11.39.7 antibody also showed some cross-reactivity to DR4, but not to DcR1 or DcR2.

#### EXAMPLE 13

20

##### Antibody Isotyping

The isotype of the 3F11.39.7 antibody (as described above) was determined by coating microtiter plates with isotype specific goat anti-mouse Ig (Fisher Biotech, Pittsburgh, PA) overnight at 4°C. The plates were then washed with wash buffer (as described in Example 9 above). The wells in the microtiter plates were then blocked with 200 µl of 2% bovine serum albumin and incubated at room temperature for one hour. The plates were washed again three times with wash buffer. Next, 100 µl of 5 µg/ml of purified 3F11.39.7 antibody was added to designated wells. The plates were incubated at room temperature for 30 minutes and then 50 µl HRP-conjugated goat anti-mouse IgG (as described above) was added to each well. The plates were incubated for 30 minutes at room temperature. The level of HRP bound to the plate was detected using HRP substrate as described above.

35 The isotyping analysis showed that the 3F11.39.7 antibody is an IgG1 antibody.

\* \* \* \* \*

40

##### Deposit of Material

The following materials have been deposited with the American



5 Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA  
(ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
pRK5-Apo-2	209021	May 8, 1997
3F11.39.7	HB-12456	January 13, 1998

10

15 This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC Section 122 and the Commissioner's rules pursuant thereto (including 37 CFR Section 1.14 with particular reference to 886 OG 638).

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The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

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The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the

5 practice of any aspect of the invention, including the best mode  
thereof, nor is it to be construed as limiting the scope of the claims  
to the specific illustrations that it represents. Indeed, various  
modifications of the invention in addition to those shown and described  
herein will become apparent to those skilled in the art from the  
10 foregoing description and fall within the scope of the appended claims.

## (1) GENERAL INFORMATION:

(i) APPLICANT: Ashkenazi, Avi  
Chuntharapai, Anon  
Kim, Kyung Jin

(ii) TITLE OF INVENTION: Apo-2 RECEPTOR

(iii) NUMBER OF SEQUENCES: 5

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Genentech, Inc.  
(B) STREET: 1 DNA Way  
(C) CITY: South San Francisco  
(D) STATE: California  
(E) COUNTRY: USA  
(F) ZIP: 94080

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5 inch, 1.44 Mb floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: WinPatin (Genentech)

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Marschang, Diane L.  
(B) REGISTRATION NUMBER: 35,600  
(C) REFERENCE/DOCKET NUMBER: P1101P1



5	Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr Arg Asn Thr	125	130	135
	Val Cys Gln Cys Glu Glu Gly Thr Phe Arg Glu Glu Asp Ser Pro	140	145	150
10	Glu Met Cys Arg Lys Cys Arg Thr Gly Cys Pro Arg Gly Met Val	155	160	165
	Lys Val Gly Asp Cys Thr Pro Trp Ser Asp Ile Glu Cys Val His	170	175	180
15	Lys Glu Ser Gly Ile Ile Ile Gly Val Thr Val Ala Ala Val Val	185	190	195
	Leu Ile Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys	200	205	210
	Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly Gly Asp	215	220	225
25	Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro Gly Ala Glu Asp	230	235	240
	Asn Val Leu Asn Glu Ile Val Ser Ile Leu Gln Pro Thr Gln Val	245	250	255
30	Pro Glu Gln Glu Met Glu Val Gln Glu Pro Ala Glu Pro Thr Gly	260	265	270
	Val Asn Met Leu Ser Pro Gly Glu Ser Glu His Leu Leu Glu Pro	275	280	285
	Ala Glu Ala Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala	290	295	300
40	Asn Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp Asp	305	310	315

5 Phe Ala Asp Leu Val Pro Phe Asp Ser Trp Glu Pro Leu Met Arg  
320 325 330

10 Lys Leu Gly Leu Met Asp Asn Glu Ile Lys Val Ala Lys Ala Glu  
335 340 345

Ala Ala Gly His Arg Asp Thr Leu Tyr Thr Met Leu Ile Lys Trp  
350 355 360

15 Val Asn Lys Thr Gly Arg Asp Ala Ser Val His Thr Leu Leu Asp  
365 370 375

Ala Leu Glu Thr Leu Gly Glu Arg Leu Ala Lys Gln Lys Ile Glu  
380 385 390

20 Asp His Leu Leu Ser Ser Gly Lys Phe Met Tyr Leu Glu Gly Asn  
395 400 405

Ala Asp Ser Ala Xaa Ser  
25 410 411

(2) INFORMATION FOR SEQ ID NO:2:

30 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1799 base pairs  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Single  
(D) TOPOLOGY: Linear

35 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCCACGCGTC CGCATAAATC AGCACGCGGC CGGAGAACCC CGCAATCTCT 50

40 GCGCCACAA AATACACCGA CGATGCCCGA TCTACTTTAA GGGCTGAAAC 100

CCACGGGCCT GAGAGACTAT AAGAGCGTTC CCTACCGCC ATG GAA 145

Met Glu

1

CAA CGG GGA CAG AAC GCC CCG GCC GCT TCG GGG GCC CGG 184  
Gln Arg Gly Gln Asn Ala Pro Ala Ala Ser Gly Ala Arg

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AAA AGG CAC GGC CCA GGA CCC AGG GAG GCG CGG GGA GCC 223  
Lys Arg His Gly Pro Gly Pro Arg Glu Ala Arg Gly Ala

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AGG CCT GGG CTC CGG GTC CCC AAG ACC CTT GTG CTC GTT 262  
Arg Pro Gly Leu Arg Val Pro Lys Thr Leu Val Leu Val

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GTC GCC GCG GTC CTG CTG TTG GTC TCA GCT GAG TCT GCT 301  
Val Ala Ala Val Leu Leu Leu Val Ser Ala Glu Ser Ala

45

50

CTG ATC ACC CAA CAA GAC CTA GCT CCC CAG CAG AGA GCG 340  
Leu Ile Thr Gln Gln Asp Leu Ala Pro Gln Gln Arg Ala

55

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GCC CCA CAA CAA AAG AGG TCC AGC CCC TCA GAG GGA TTG 379  
Ala Pro Gln Gln Lys Arg Ser Ser Pro Ser Glu Gly Leu

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TGT CCA CCT GGA CAC CAT ATC TCA GAA GAC GGT AGA GAT 418  
Cys Pro Pro Gly His His Ile Ser Glu Asp Gly Arg Asp

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TGC ATC TCC TGC AAA TAT GGA CAG GAC TAT AGC ACT CAC 457  
Cys Ile Ser Cys Lys Tyr Gly Gln Asp Tyr Ser Thr His

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TGG AAT GAC CTC CTT TTC TGC TTG CGC TGC ACC AGG TGT 496  
Trp Asn Asp Leu Leu Phe Cys Leu Arg Cys Thr Arg Cys

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GAT TCA GGT GAA GTG GAG CTA AGT CCC TGC ACC ACG ACC 535  
 Asp Ser Gly Glu Val Glu Leu Ser Pro Cys Thr Thr Thr  
 120 125 130

10

AGA AAC ACA GTG TGT CAG TGC GAA GAA GGC ACC TTC CGG 574  
 Arg Asn Thr Val Cys Gln Cys Glu Glu Gly Thr Phe Arg  
 135 140 145

15

GAA GAA GAT TCT CCT GAG ATG TGC CGG AAG TGC CGC ACA 613  
 Glu Glu Asp Ser Pro Glu Met Cys Arg Lys Cys Arg Thr  
 150 155

20

GGG TGT CCC AGA GGG ATG GTC AAG GTC GGT GAT TGT ACA 652  
 Gly Cys Pro Arg Gly Met Val Lys Val Gly Asp Cys Thr  
 160 165 170

25

CCC TGG AGT GAC ATC GAA TGT GTC CAC AAA GAA TCA GGC 691  
 Pro Trp Ser Asp Ile Glu Cys Val His Lys Glu Ser Gly  
 175 180



5 ATC ATC ATA GGA GTC ACA GTT GCA GCC GTA GTC TTG ATT 730  
 Ile Ile Ile Gly Val Thr Val Ala Ala Val Val Leu Ile  
 185 190 195

10 GTG GCT GTG TTT GTT TGC AAG TCT TTA CTG TGG AAG AAA 769  
 Val Ala Val Phe Val Cys Lys Ser Leu Leu Trp Lys Lys  
 200 205 210

15 GTC CTT CCT TAC CTG AAA GGC ATC TGC TCA GGT GGT GGT 808  
 Val Leu Pro Tyr Leu Lys Gly Ile Cys Ser Gly Gly Gly  
 215 220

20 GGG GAC CCT GAG CGT GTG GAC AGA AGC TCA CAA CGA CCT 847  
 Gly Asp Pro Glu Arg Val Asp Arg Ser Ser Gln Arg Pro  
 225 230 235

25 GGG GCT GAG GAC AAT GTC CTC AAT GAG ATC GTG AGT ATC 886  
 Gly Ala Glu Asp Asn Val Leu Asn Glu Ile Val Ser Ile  
 240 245

30 TTG CAG CCC ACC CAG GTC CCT GAG CAG GAA ATG GAA GTC 925  
 Leu Gln Pro Thr Gln Val Pro Glu Gln Glu Met Glu Val  
 250 255 260

35 CAG GAG CCA GCA GAG CCA ACA GGT GTC AAC ATG TTG TCC 964  
 Gln Glu Pro Ala Glu Pro Thr Gly Val Asn Met Leu Ser  
 265 270 275

40 CCC GGG GAG TCA GAG CAT CTG CTG GAA CCG GCA GAA GCT 1003  
 Pro Gly Glu Ser Glu His Leu Leu Glu Pro Ala Glu Ala  
 280 285

GAA AGG TCT CAG AGG AGG AGG CTG CTG GTT CCA GCA AAT 1042  
 Glu Arg Ser Gln Arg Arg Arg Leu Leu Val Pro Ala Asn  
 290 295 300

GAA GGT GAT CCC ACT GAG ACT CTG AGA CAG TGC TTC GAT 1081  
 Glu Gly Asp Pro Thr Glu Thr Leu Arg Gln Cys Phe Asp

310

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78



5 (2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: Nucleic Acid

10 (C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

15

ATCAGGGACT TTCCGCTGGG GACTTTCCG 29

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGATGGGAA GTGTGTGATA TATCCTTGAT 30

What is claimed is:

1. An antibody which specifically binds to Apo-2.
2. The antibody of claim 1 which is a monoclonal antibody.
3. The antibody of claim 1 which is an agonist antibody.
4. The antibody of claim 1 which is a blocking antibody.
5. The antibody of claim 1 which is a chimeric antibody.
6. A hybridoma cell line which produces the antibody of claim 2.
7. The antibody of claim 2 having the biological characteristics of the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456.
8. The antibody of claim 2 wherein the antibody binds to the same epitope as the epitope to which the monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456 binds.
9. The hybridoma cell line deposited under American Type Culture Collection Accession Number HB-12456.
10. The monoclonal antibody produced by the hybridoma cell line deposited under American Type Culture Collection Accession Number ATCC HB-12456.
11. An isolated nucleic acid encoding the Apo-2 antibody of claim 1.
12. A composition comprising the antibody of claim 1 and a carrier.
13. The composition of claim 12 wherein said carrier is a pharmaceutically acceptable carrier.
14. A method of inducing apoptosis in mammalian cells comprising

exposing mammalian cells to an effective amount of an Apo-2 agonist antibody.

15. An article of manufacture, comprising a container and a composition contained within the container, wherein the composition includes an Apo-2 antibody.

16. The article of manufacture of claim 15 further comprising instructions for using the Apo-2 antibody *in vivo* or *in vitro*.

17. A dimeric molecule comprising an Apo-2 antibody linked to a heterologous immunoglobulin.

18. A homodimeric molecule comprising a first Apo-2 antibody and a second Apo-2 antibody.

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Abstract of the Disclosure

Novel polypeptides, designated Apo-2, which are capable of modulating apoptosis are provided. Compositions including Apo-2 chimeras, nucleic acid encoding Apo-2, and antibodies to Apo-2 are also provided.

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2000-04-20

Fig. 1

1	CCCACGGCTC	CGCATAAATC	AGCACGGGC	CGGAGAACCC	CGCAATCTCT	GCGCCACAA	AATACACCGA	CGATGCCCGA	TCTACTTTAA	GGGCTGAAAC
	GGGTGGCAG	GGGTATTTAG	TCGTGGCCG	GCCTCTGGG	GCCTTAGAGA	CGCGGTGTT	TTATGTGGCT	GCTACGGGCT	AGATGAAATT	CCCGACTTTG
101	CCACGGGCTT	GAGAGACTAT	AAGAGCGTTC	CCTACCGCCA	TGGAACAACG	GGACAGAAC	CCCCCGGGC	CTTCGGGGG	CCGGAAAAGG	CACGGCCCCAG
	GGTGGCCGGA	CTCTCTGATA	TTCTCGCAAG	GGATGGCGGT	ACCTTGTTGC	CCCTGTCTTG	CGGGCCCGG	GAAGCCCCCG	GGCCTTTTC	GTGCGGGTC
1					M etGluGlnAr	gGlyGlnAsn	AlaProAla	laserGlyAl	aArgLysArg	HisGlyProGly
201	GACCCAGGGA	GGCGGGGGA	GCCAGGCCCTG	GGTCCGGGT	CCCCAAGACC	CTTGTGCTCG	TTGTGCGCGC	GGTCTGCTG	TTGGTCTCAG	CTGAGTCTGC
	CTGGGTCCCT	CCGCGCCCT	CGGTCCGGAC	CCGAGGCCCA	GGGTCTCTGG	GAACACGAGC	AACAGCGCGC	CCAGGACGAC	AACACAGATC	GACTCAGACG
22	ProArgG1	uAlaArgGly	AlaArgProG	lyLeuArgva	lProLysThr	LeuValLeu	alValAlaAl	aValLeuLeu	LeuValserA	laGluSerAla
301	TCGTGATCAC	CAACAAGACC	TAGTCCCCA	GCAGAGAGCG	GGCCCAACA	AAAAGAGGTC	CAGCCCCCTCA	GAGGGATTGT	GTCCACCTGG	ACACCATATC
	AGACTAGTGG	GTTGTTCTGG	ATCGAGGGGT	CGTCTCTCGC	CGGGGTGTTG	TTTTCTCCAG	GTGCGGGAGT	CTCCCTAACA	CAGGTGGACC	TGTGGTATAG
55	LeuileThr	GlnGlnAspL	euAlaProG1	nGlnArgAla	AlaProGlnG	lnLysArgse	rserProser	gluglyLeuC	ysProProG1	yHisHisile
401	TCAGAAGACG	GTAGAGATTG	CATCTCCTGC	AAATATGGAC	AGGACTATAG	CACCTCACTGG	AATGACCTCC	TTTTCTGCTT	GGCTGCACC	AGGTGTGATT
	AGTCTTCTGC	CATCTCTAAC	GTAGAGGACG	TTTATACCTG	TCCTGATATC	GTGAGTGACC	TTACTGGAGG	AAAAGACGAA	CGCGACGTGG	TCCACACTAA
88	SerGluAspG	lyArgAspCy	sileserCys	LystyrGlyG	lnAspTyse	rThrHisTrp	AsnAspLeuL	eupheCysLe	uArgCysThr	ArgCysAspSer
501	CAGGTGAAGT	GGAGCTAAGT	CCCTGCACCA	CGACCAGAAA	CACAGTGTGT	CAGTGCGAAG	AAGGCACCTT	CCGGGAAGAA	GATTCTCCTG	AGATGTGCCG
	GTCCACITCA	CCTCGATTCA	GGGACGTGGT	GCTGGTCTTT	GTGTACACA	GTACACGCTT	TTCCGTGGA	GGCCTTCTT	CTAAGAGGAC	TCTACACGGC
122	GlyGluva	lGluLeuser	ProCysThrt	hrThrArgAs	nThrValCys	GlnCysGluG	luglyThrPh	eArgGluGlu	AspSerProG	luMetCysArg
601	GAAGTGCCG	ACAGGGTGT	CCAGAGGGAT	GGTCAAGGTC	GGTGATTGTA	CACCTTGGAG	TGACATCGAA	TGTGTCCACA	AAGAAATCAGG	CATCATCATA
	CTTCACGGCG	TGTCCACACG	GGTCTCCCTA	CCAGTTCCAG	CCACTAACAT	GTGGGACCTC	ACTGTAGCTT	ACACAGGTGT	TTCTTAGTCC	GTAGTAGTAT
155	LysCysArg	ThrGlyCysP	roArgGlyMe	tvallysVal	GlyAspCyst	hrProTrpse	raspileGlu	CysValHisL	ysGluSerG1	yileille
701	GGAGTCACAG	TTGCAGCCGT	AGTCTTGATT	GTGGCTGTGT	TTGTTTGCAA	GTCTTTACTG	TGGAAGAAAG	TCCTTCCCTTA	CCTGAAAAGG	ATCTGCTCAG
	CCTCAGTGT	AAGTCCGGA	TCAGAACTAA	CACCGACACA	AACAAACGTT	CAGAAATGAC	ACCTTCTTTC	AGGAAGGAAT	GGACTTTCG	TAGACGAGTC
188	GlyvalThr	alAlaAla	lvalleuile	valAlaValP	heValCysLy	sSerLeuLeu	TrpLysLysv	alleuProTy	rLeuLysGly	ileCysSerGly
801	GTGGTGGTGG	GGACCCCTGAG	CGTGTGGACA	GAAAGCTCACA	ACGACCTGGG	GCTGAGGACA	ATGTCCCTCAA	TGAGATCGTG	AGTATCTTGC	AGCCCAACCCA
	CACCAACACC	CCTGGGACTC	GCACACCTGT	CTTCGAGTGT	TGCTGGACCC	CGACTCCTGT	TACAGGAGTT	ACTCTAGCAC	TCATAGAACG	TCGGGTGGGT
222	GlyGlyG1	yaspproGlu	ArgvalAspA	rgSerSerG1	ProThrGlyv	AlaGluAspA	snValLeuAs	nGluileVal	SerilleLeuG	lnProThrGln
901	GGTCCCTGAG	CAGGAAATGG	AAGTCCAGGA	GCCAGCAGAG	CCAACAGGTG	TCAACATGTT	GTCCCCCGGG	GAGTCAGAGC	ATCTGCTGGA	ACCGGCAGAA
	CCAGGGACTC	GTCCTTTACC	TTCAGGTCTC	CGGTCTCTC	GGTTGTCCAC	AGTTGTACAA	CAGGGGGCCC	CTCAGTCTCG	TAGACACCT	TGGCCGTCTT
255	ValProGlu	GlnGluMetG	luValGlnG1	uProAlaGlu	ProThrGlyv	alAsnMetLe	userProGly	GluSerGluH	isLeuLeuG1	uProAlaGlu
1001	GCTGAAAGGT	CTCAGAGGAG	GAGGCTGCTG	GTTCACGCAA	ATGAAGGTGA	TCCCACCTGAG	ACTCTGAGAC	AGTGCTTCCA	TGACTTTTGA	GACTTGGTGC
	CGACTTTCCA	GAGTCTCTC	CTCCGACGAC	CAAGGTCTGT	TACTTCCACT	AGGTGACTC	TGAGACTCTG	TCACGAAGCT	ACTGAAACGT	CTGAACCCAG
288	AlaGluArgS	erGlnArgar	gArgLeuLeu	ValProAla	snGluGlyAs	pProThrGlu	ThrLeuArgG	lnCysPheAs	paspPheAla	AspLeuvalPro



1101 CCTTTGACTC CTGGGAGCCG CTCATGAGGA AGTTGGGCGCT CATGGACAAT GAGATAAAGG TGGCTAAAGC TGAGGCAGCG GCCACACAGG ACACCTTGTA  
GGAACCTGAG GACCCCTGGC GAGTACTCCT TCAACCCGGA GTACCTGTTA CTCATTTTCC ACCGATTTCG ACTCGTCCG CCGGTGTCCQ TGTGGAACAT  
322 PheAspse rTrpGluPro LeuMetArgL ysLeuGlyLe uMetAspAsn GluileLysV alAlaLysAl aGluAlaAla GlyHisArga sPThrLeutyf  
1201 CACGATGCTG ATAAAGTGGG TCAACAAAAC CGGGCGAGAT GCCTCTGTCC ACACCCTGCT GGATGCCCTTG GAGACGCTGG GAGAGAGACT TGCCAAGCAG  
GTGCTACGAC TATTTCAACC AGTTGTTTTG GCCCGCTCTA CGGAGACAGG TGTGGGACGA CCTACGGAAC CTCCTCGACC CTCTCTCTGA ACGTTCGTC  
355 ThrMetLeu IleLysTrpV alAsnLysTh rGlyArgAsp AlaSerValH iSThrLeule uAspAlaLeu GluThrLeug lyGluArgLe uAlaLysGln  
1301 AAGATTGAGG ACCACTTTGT GAGCTCTGGA AAGTTTCATGT ATCTAGAAGG TAATGCAGAC TCTGCCWGTG CCTAAGTGTG ATTCTCTTCA GGAAGTGAGA  
TTCTAACTCC TGGTGAACAA CTCGAGACCT TTCAAGTACA TAGATCTTCC ATTACGTCTG AGACGGAAAC GGATTTCACAC TAAGAGAAAGT CCTTCACTCT  
388 LysileGluA sPHisLeule uSerSerGly LysPheMetT yrLeuGluGl yAsnAlaAsp SerAlaXqqS eroC\*  
1401 CCTTCCCTGG TTTACCTTTT TTCTGGAAAA AGCCCAACTG GACTCCAGTC AGTAGGAAAG TGCCACAATT GTCACATGAC CGGTACTGGA AGAAACTCTC  
GGAAGGGACC AAATGGAAAA AAGACCTTTT TCGGGTTGAC CTGAGGTCAG TCATCCTTTC ACGGTGTAA CAGGTACTG GCCATGACCT TCTTTGAGAG  
1501 CCATCCAAAC TCACCCAGTG GATGGAACAT CCTGTAACTT TTCACTGCAC TTGGCATTAT TTTTATAAGC TGAATGTGAT AATAAGGACA CTATGGAAAT  
GGTAGGTTGT AGTGGGTCAC CTACCTTGTA GGACATTGAA AAGTGACGTG AACCGTAATA AAAATATTCTG ACTTACACTA TTATTCTGT GATACCTTTA  
1601 GTCTGGATCA TTCCGTTTGT GCGTACTTTG AGATTGTGTT TGGGATGTCA TTGTTTTCAC AGCACTTTT TATCCTAATG TAAATGCTTT ATTTATTTAT  
CAGACCTAGT AAGGCAACAC CGCATGAAAC TCTAAACCAA ACCCTACAGT AACAAAAAGTG TCGTGAAAAA ATAGGATTAC ATTTACGAAA TAAATAAATA  
1701 TTGGGGCTACA TTGTAAGATC CATCTACAAA AAAAAAAA AAAAAAAA GGGGGCCGCG ACTCTAGAGT CGACCTGCGA AAGCTTGGCC GCCATGGCC  
AACCCGATGT AACATTCTAG GTAGATGTTT TTTTTTTTTT TTTTTTTTTT CCGCCGGCGC TGAGATCTCA GCTGGACGTC TTCGAAACCG CGGTACCGG

Fig. 1 (cont.)

Fig. 2 A

1 MEORGONAPAA SGARKRHGPGPREARGARGLRVPKTLVLVAAVLLLVSAESALITQQD  
 61 LAPQORAAFPQOKRSSPSEGLCPPGHHISEDGRDCISCKYQDYSTHWNDDLFLRLCTRCD  
 121 SGEVELSPCTTTRNTVCQCEGTFREEDSPENCRKCRGCPRGVMKVGDCTPWSDIECVH  
 181 KESGLIIGVTAAAVLIVAVFVCKSLMKKVLPLYLKIGICSGGGGDPERVDRSSQRPGAED  
 241 NVLNEIVSILOPTQVPEQEMEVEQPEAPETGVNMLSPGESEHLLLEPAEAERSQRRLLVPA  
 301 NEGDPTEILRQCDDFADILVPFDSWEPLMRKIGLMDNEIKVAKAEAAAGHRDTLYTMLIKW  
 361 VNKTGRDASVHTLLDALETLGERLAKQKIEDHLLSSGKFMYLEGNADSALS

Fig. 2 B

Apo2	FADLVPPFDSWEPIMRKIGIMDNEIKVAKAEAA--CHRD <sup>*</sup> TI
DR4	FANIVPPFDSWDQILMRQLDLTKNEIDVVRA <sup>*</sup> GTA--GPGDAL
Apo3/DR3	VMBAVPPARRWK <sup>*</sup> EFV <sup>*</sup> RTLGLEAEIEAVEVEIGR--FRDQQ
TNFR1	VVENVPPLRWK <sup>*</sup> EFV <sup>*</sup> RRIGLSDHEIDRLLELQNGR-CLREAQ
Fas/Apo1	IRGVMTLSQVKG <sup>*</sup> FVRKNGVN <sup>*</sup> EAKIDEIKNDNVQDTAEQKV

Apo2	YTMLIK <sup>*</sup> WV <sup>*</sup> NK <sup>*</sup> TGRD-ASVHTLLD <sup>*</sup> DALETLGERLAKQKIED
DR4	YAMLMK <sup>*</sup> WV <sup>*</sup> NK <sup>*</sup> TGRN-ASVHTLLD <sup>*</sup> DALE <sup>*</sup> ERMEE <sup>*</sup> RHAK <sup>*</sup> EKI <sup>*</sup> QD
Apo3/DR3	YEMLK <sup>*</sup> RR <sup>*</sup> W <sup>*</sup> RQQP--AGLGAVYAALERMGLDGCVE <sup>*</sup> DLRS
TNFR1	YSMLAT <sup>*</sup> W <sup>*</sup> RR <sup>*</sup> RT <sup>*</sup> PR <sup>*</sup> RE <sup>*</sup> ATLELGRVDRMDLLGCL <sup>*</sup> E <sup>*</sup> DEE
Fas/Apo1	-QLLRN <sup>*</sup> WH <sup>*</sup> QL <sup>*</sup> HG <sup>*</sup> KK <sup>*</sup> EAY-DT <sup>*</sup> LIK <sup>*</sup> DDL <sup>*</sup> KKAN <sup>*</sup> CTLA <sup>*</sup> EKI <sup>*</sup> QT

Fig. 3

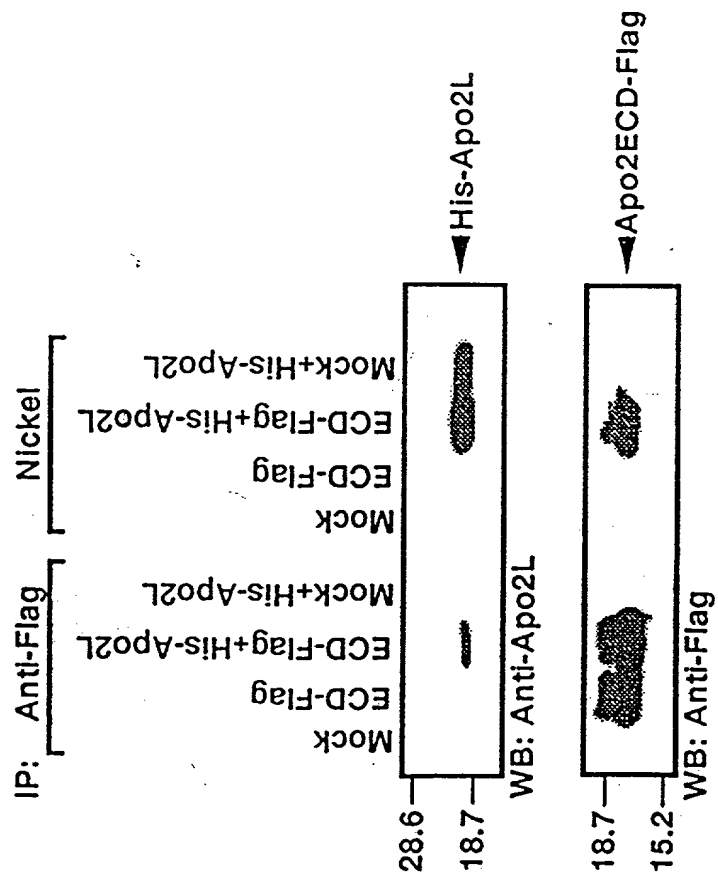
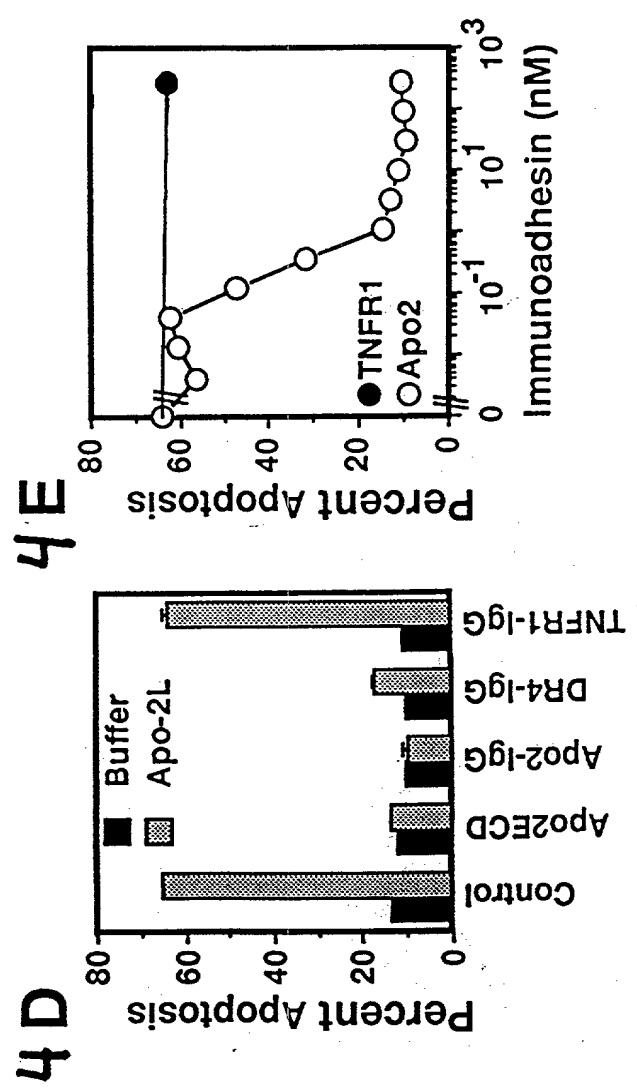
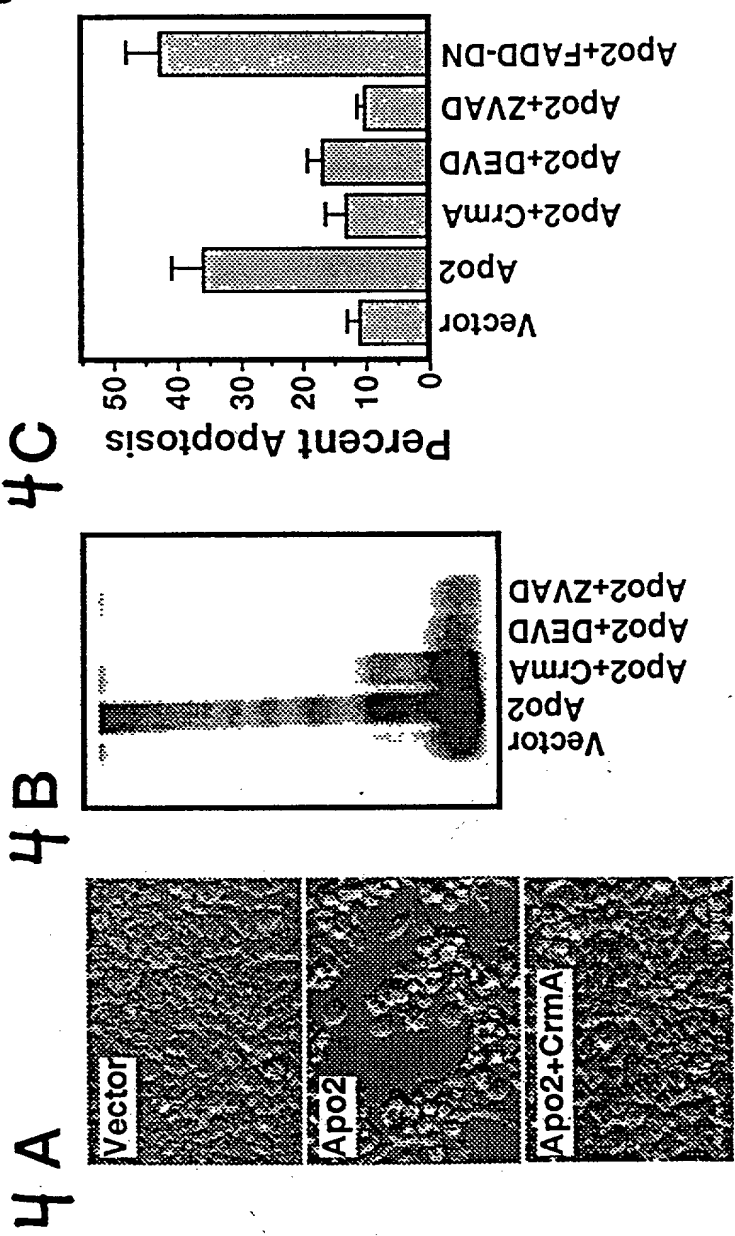
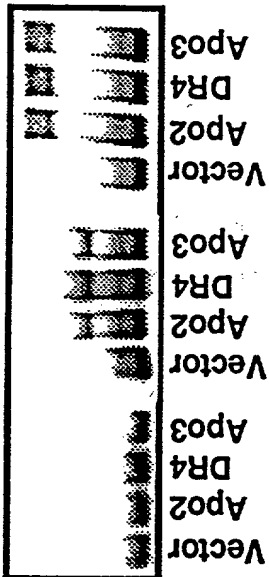


Fig. 4

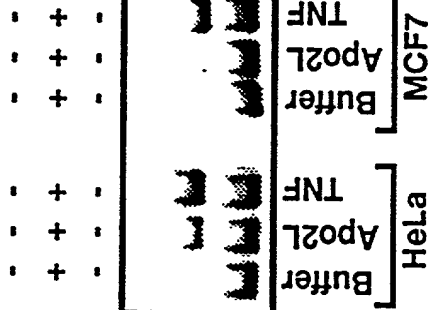


5 A

Unlabelled probe  
Labelled probe  
Anti-p65



5 B



5 C

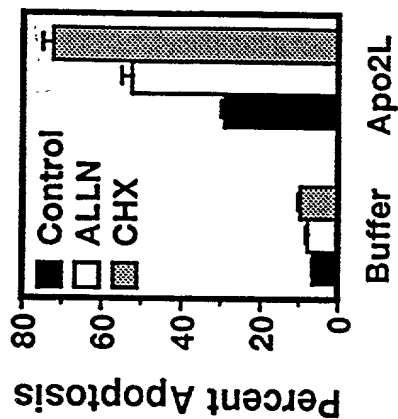
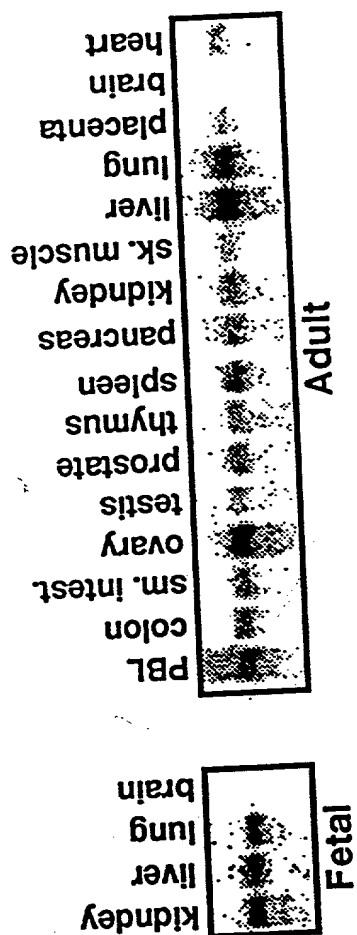


Fig. 5

FIG. 6



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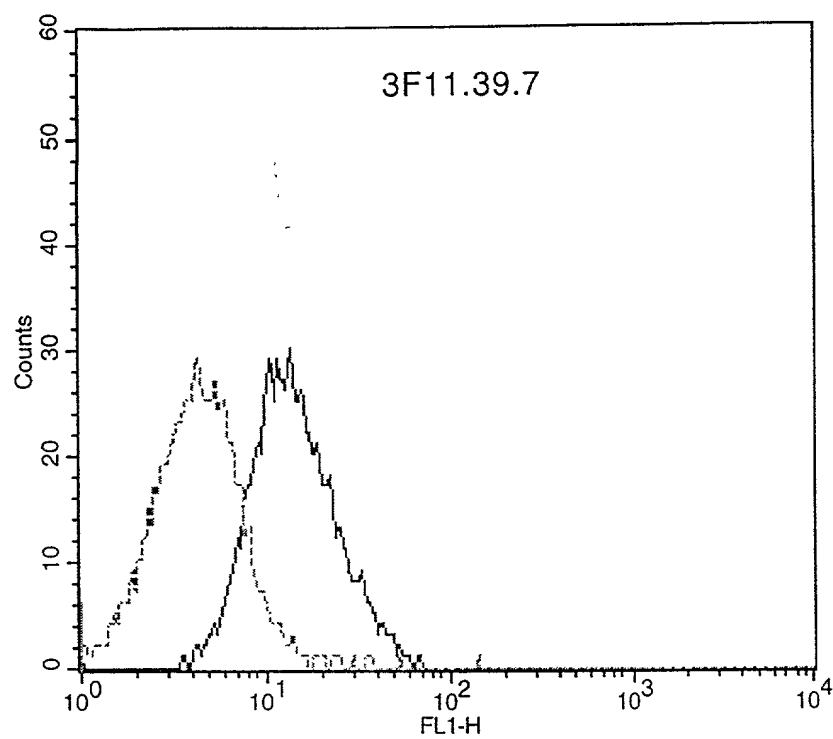


Fig. 7

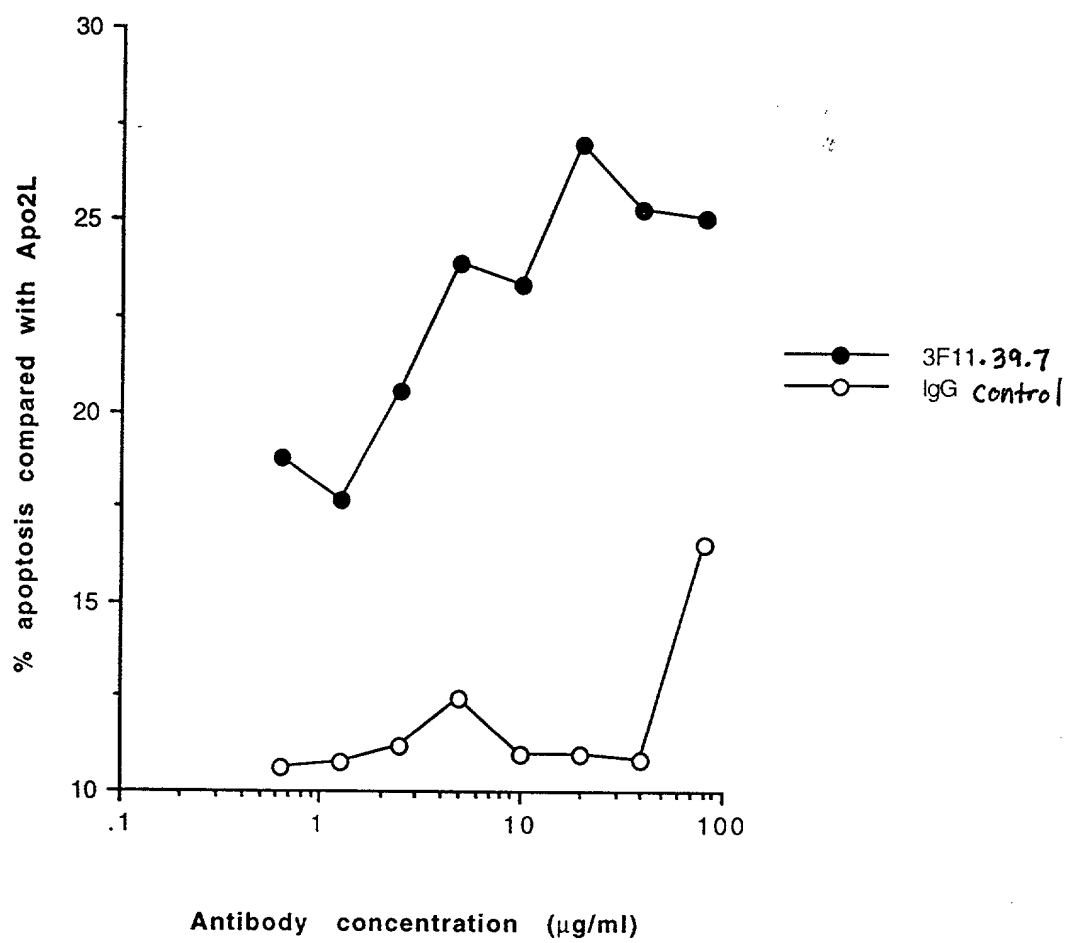


Fig. 8



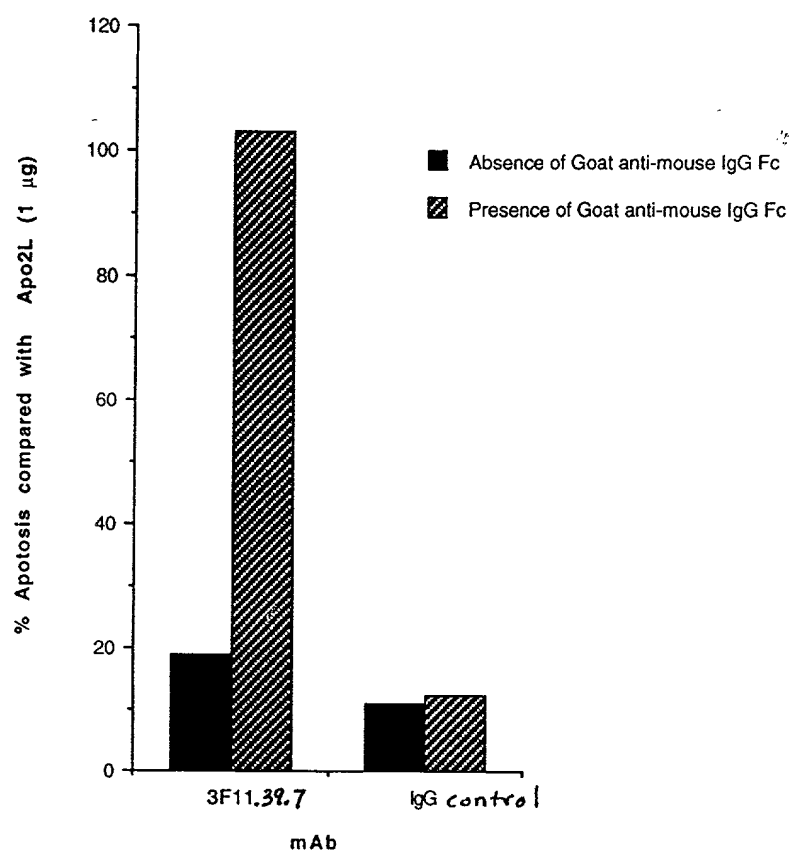


Fig. 9

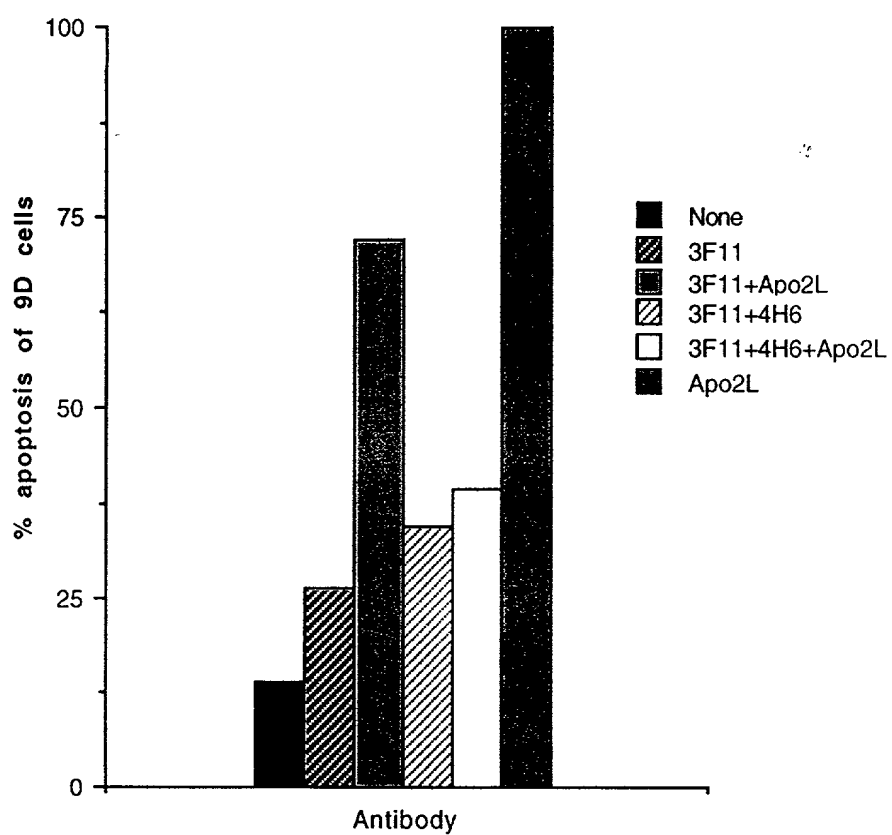


Fig. 10

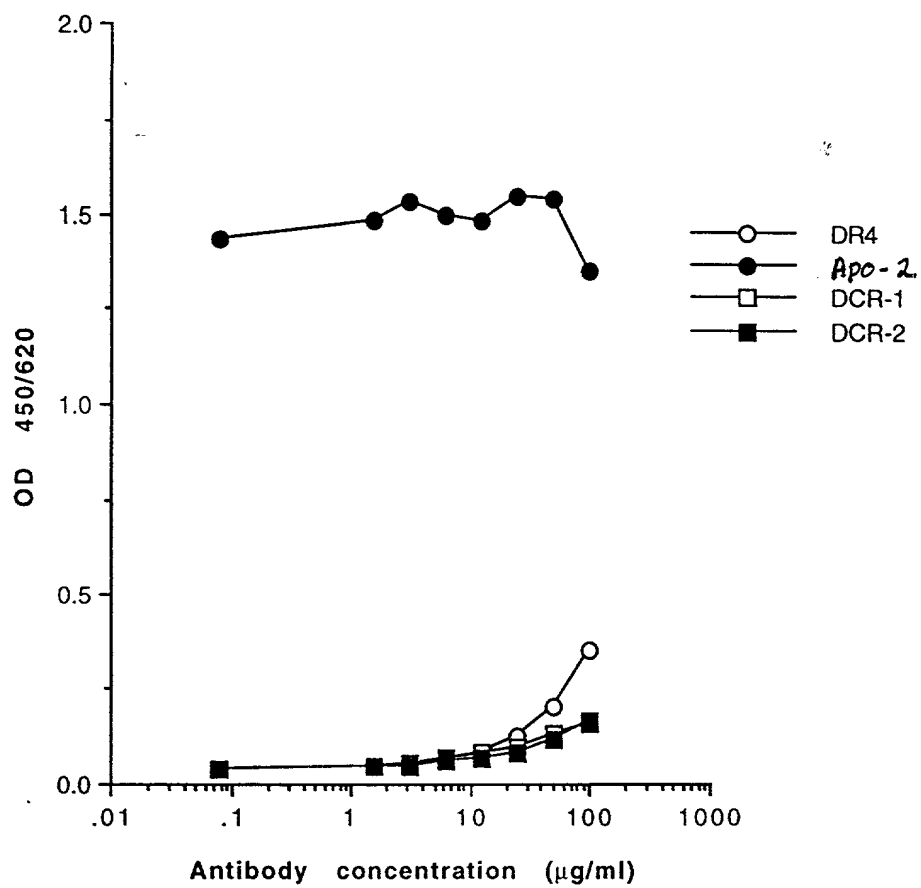
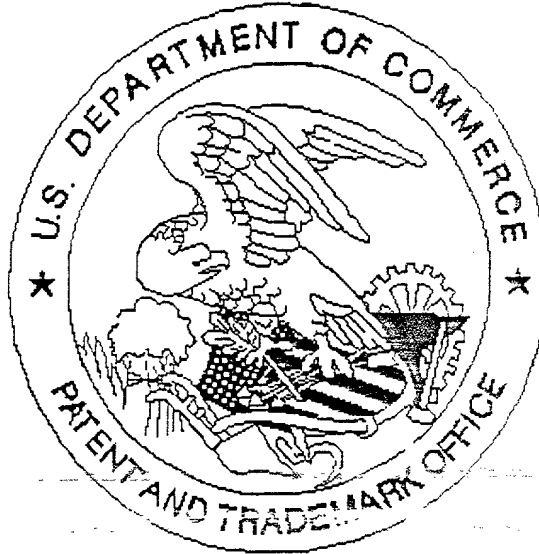


Fig. 11

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